SURVIVABILITY OF TOTAL COLIFORMS IN

FREEZING AND FROZEN SOILS

By

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A

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Abstract

The study showed that a significant fraction of coliform bacteria survive for more than six months in soil at different temperatures and moisture contents. Survivability of coliform bacteria at subzero temperatures decreased with an increase in moisture content and with an increase in temperature. Total coliform bacteria in soil samples placed outdoors during winter had lower survivability in comparison to samples placed at controlled temperatures below $0^{o}C$. High survivability of total coliform bacteria at controlled, subzero temperatures was assumed to be related to the reduced metabolic activities of the bacteria.

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Chapter One

Introduction

1.1. Background

In rural communities of Alaska, there are no running water supplies and thus no flush toilets. Human waste is collected in containers, known as honey buckets, and disposed of in dumps. Dumpsites in rural communities receive less maintenance when compared to those of larger population centers, mainly due to economic reasons. Solid waste management in rural Alaskan communities can include open burning and applying of cover soils. However, even with these management operations, limiting the spread of human waste is difficult.

Human excreta are considered to be the principal vehicle for the transmission and spread of a wide range of communicable diseases. Pathogens in the excreta of an infected individual transmit to a new victim through direct or indirect pathways. Direct contact with pathogens may occur through contact with honey buckets due to leakage during hauling from the household to the dumpsites. Uncovered honey buckets with excreta increases the possibilities of people being in contact with fecal matter every time the honey bucket is used. In rural community dumpsites, waste is occasionally left at the entrance, increasing the possibility of human contact with excreta. In some cases, waste is disposed of outside the dump boundaries, increasing the chances of human contact as others transport their wastes to the dump or by children playing outside the dumpsite boundaries.

Indirect transmission of pathogens from excreta to healthy persons occurs through vectors. Vectors can include vehicles, humans or animals commuting from the contaminated sites, such as dumpsites, onto the roads and into the houses where food and water can be contaminated (Chambers et al., 2005). Wind may also blow contaminated trash from the dumpsites to the communities. Water flowing from contaminated sites during rains or spring snow melt may carry pathogens and contaminate water sources such as lakes or rivers. Exposed solid waste may attract animals or birds, resulting in the transport of pathogens from dumpsites into water sources or into communities. Other disease vectors such as rats, mosquitoes and flies breeding at the dumpsites may also transmit diseases into the communities.

1.2. Problem statement

Human excreta disposed openly in the environment may harbor pathogens. Subzero temperatures and snow cover dominate many Alaskan communities during winter months, decreasing the probability of pathogen transmission. The decrease in human and animal outdoor activities in the extreme low temperatures also reduces the probability of transmission of pathogens. During spring thaw, however, runoff from snow melt harboring pathogens may flow from the dump area towards the community or towards drinking water sources.

Even though transmission and survival characteristics of pathogens, as indicated by indicator organisms, is an intensively studied phenomenon for different environmental conditions (Boyd et al., 1969; Chandler and Craven, 1978; Cuthbert et al., 1950; Klein

and Casida, 1967; Mallman and Litsky, 1951; Tate 1978), there have been few studies on pathogen survival in arctic and subarctic climates. This study examines survivability of coliform bacteria in the soil at different subzero temperatures and moisture levels. Results from this study will aid in management of human excreta and communicable diseases in rural arctic and subarctic communities.

1.3. Objective and hypothesis

The living environment for the microorganisms in human excreta changes considerably when honey bucket wastes are dumped into the dumpsite and infiltrate into surface soil. Survivability of microorganisms in the soil environment depends on the individual organisms. Temperature, moisture content and pH of the soil are a few of the factors that determine the survivability of microorganisms in soil (Reddy et al., 1981). While others have studied microorganisms in soil under different conditions (Boyd et al., 1969; Chandler and Craven, 1978; Cuthbert et al., 1950; Klein and Casida, 1967; Mallman and Litsky, 1951; Tate 1978), limited information is available on the survivability of coliforms in freezing soil at different moisture contents. The objective of this study was to find out how long fecal contamination indicators (i.e. total coliforms) survive at different temperatures and different moisture contents. The survivability of coliform in the soil was then related to the survivability of pathogens that originated and live in an environment similar to that of coliform bacteria.

The hypothesis for the study was:

Survivability of coliform bacteria in frozen soil depends on pre-freezing moisture content and temperature.

To test the hypothesis, samples containing coliforms were prepared by mixing soils with dog fecal matter at moisture contents of 24%, 37% and 49% with respect to the dry mass of the soil. Prepared soil samples were placed outdoors at an uncontrolled temperature or at different controlled, constant temperatures of 20°C, -5°C,-15°C, -20°C and -28°C, for at least 170 days. At different time intervals, soil samples were tested for the presence of total coliform bacteria. Detailed procedures for preparing soil samples and population enumeration are discussed in Chapter Three.

Results from the study indicate that coliforms can survive in a subzero soil environment for more than one hundred and seventy days. Survivability was found to be more at subzero temperatures than at room temperature. Thus, coliforms introduced into the soil before winter are likely to survive, along with fecally transmittable pathogens, throughout the winter and may be transported by runoff during the spring thaw.

Chapter Two

Literature Review

2.1. Coliform as an indicator of fecal contamination

Water is a common medium for disease transmission. Thus it is necessary to use water which is free from pathogenic microorganisms and harmful chemicals. It may not be practical to identify individual microorganisms present in water. However, it is practical to sample water supplies for overall presence of microorganisms (Madigan et al., 2000). Some non-pathogenic microorganisms thriving on a particular host can be taken as an indicator of the presence of fecal contamination. The presence of fecal contamination could indicate the presence of human pathogens.

Fecal contamination indicator bacteria for water supply testing are selected from among the microorganisms which flourish in the intestinal tract of healthy warm blooded animals. In order to be good indicators, these bacteria must to be large in numbers, easy to count, unable to grow outside the intestines and not harmful for the personnel who are handling them. They are supposed to live for longer durations than the pathogens of similar origin (Feachem et al., 1983). None of the group of fecal microorganisms completely satisfies these requirements, but some of the groups satisfy more requirements than others. Common indicators of fecal contamination are coliforms, fecal streptococci and an anaerobic bacterium *Clostridium perfringens*. Among these, total coliforms and fecal coliforms are the most commonly tested indicators.

Coliforms belong to the Enterobacteriaceae family and measure approximately 2 to 5 μm in length by 0.5 μm in width. They are gram negative, non-spore forming, rod shaped bacteria that can ferment lactose and produce gas within 48 hours at 35°C (Madigan et al., 2000; Plews et al., 1985). The total coliform group includes all fecal coliforms. Approximately 90% of the coliforms in fresh feces of warm blooded animals are *Escherichia coli* (*E. coli*) and the remainder is mainly *Citrobacter*, *Enterobacter* and *Klebsiella.* The former are exclusively fecal in origin, whereas the latter also thrive naturally in unpolluted soils and water.

Fecal coliforms have two phases of habitat which are the in-host environment (intestinal tract) and non-host environment. The in-host environment provides the optimum conditions for fecal coliforms to thrive. The temperature is constant and warm, and they have ready access to nutrients (Savageau, 1983). Coliform bacteria face severe changes in the environment when they are excreted by the host. In the non-host environment, lack of sufficient nutrients, variation in temperature and pH, change in osmotic pressure and the threat from predators lead to the restriction of growth of the coliform population (Rozen et al., 2001; Savageau, 1983). Due to the environmental stresses, cells may become viable but non culturable (McDougald et al., 1998; Winfield and Groisman, 2003). However, viable but non culturable cells may resuscitate if ingested by a host (McDougald et al., 1998; Ravel et al., 1995; Smith et al., 1994). If suitable environment and sufficient nutrient is available in the non-host environment, then net increase in population may also occur (Byappanahalli and Fujioka, 1998; Gerba and McLeod, 1976).

Fecal coliforms such as *E.coli* do not generally cause severe disease (Plews et al., 1985) but some strains of *E. coli,* such as *E. coli* O157, cause haemorrhagic colitis, haemolytic uremic syndrome, and occasionally mild non-bloody diarrhea (Chapman et al., 1997; Jones, 1999; Sack et al., 1975). These bacteria are found in the intestinal tract of humans as well as animals, indicating both humans and animals as potential sources. The coliform bacteria and pathogens behave similarly in the water environment but the coliforms do not die at a faster rate than certain pathogenic bacteria such as *Salmonella* and *Shigella* (Madigan et al., 2000).

Fecal coliforms are generally the preferred fecal contamination indicator. However, total coliform is a suitable indicator as well. Viable total coliforms present in drinking water indicate insufficient disinfection. Hence, the United States Environmental Protection Agency (EPA) mandates total coliform testing at sites which are representative of water quality throughout the distribution system. For each total coliform positive result from routine sampling of water supply system, the EPA requires a test for the presence of fecal coliform or *E. coli*. If any routine sample is total coliform positive, at least three repeat samples are required to be taken within 24 hours (EPA, 1989). This requirement shows the importance of testing of coliform and its acceptance as fecal contamination indicator.

2.2: Factors affecting survivability of coliforms in soil

Survivability of coliform in the non-host environment depends upon several factors and survival times vary widely. Two or more factors may be affecting the

survivability at the same time. The wide variation of die-off rates for coliform bacteria was tabulated by Reddy et al. (1981), after analyzing the data obtained from previous studies conducted by different researchers. Some of the major factors that affected the die-off rate of coliforms were as following: solar radiation, pH of the soil, availability of nutrients and carbon sources, moisture content in the soil, type of soil, presence of competitive microorganisms, and temperature.

Coliforms exposed to solar radiation had higher die-off rate than those which were not exposed (Sarikaya and Saatci, 1995). Neutral pH in the soil generally helped survival and growth of enteric bacteria such as coliforms. Coliforms survived better in the pH range between 6.0 and 7.0 (Ellis and McCalla, 1976; McFeters and Stuart, 1972). Cuthbert et al. (1950) showed that survivability of bacterium coli in peat moor soil with pH between 2.9 to 4.5 was much less when compared to the survivability in limestone moor soil with pH ranging 5.8 to 7.8. McFeters and Stuart (1972) measured a gradual increase in the half life of *E. coli* in deionized distilled water with an increase in pH from 2 to 7, but a gradual decrease in half life beyond pH 7.

Starvation is another principal stress that coliforms encounter in non-host environments. When an external source of nutrients is not available, microorganisms undergo endogenous metabolism by using carbon reserves in the cell (Dawes and Senior, 1973) or by using basic cell components. The slow degradation of endogenous substrate and low oxygen consumption rate positively correlates with an increase in the viability of soil microbes during starvation (Nelson and Parkinson, 1978). An increase in organic content in soil increases the available substrate for the microorganisms, which ultimately

increases the survivability of coliform (Klein and Casida, 1967; Mallman and Litsky, 1951).

The survivability of coliforms in sediment was found to be higher than in overlying marine water (Davies et al., 1995 and Gerba and McLeod, 1976) or fresh water (Davies et al., 1995; Gary and Adams, 1985 and Sherer et al., 1992). Weiss (1951) showed that *E. coli* readily adsorbs to silt found in estuaries. Coliforms were capable of using trapped and adsorbed nutrients in the sediments (Gerba and McLeod, 1976; Sherer et al., 1992) as well as the dissolved nutrients. Thus, they are likely to survive longer in a water sediment environment than in water only. In shallow water, sediment particles are also likely to provide protection from the sunlight, reducing the possibility of UV oxidation. However, the water-sediment interface is not always static. Coliforms are easily resuspended in water if the water-sediment interface is disturbed (Gary and Adams, 1985; Sherer et al. 1988).

Moisture content is a very important factor in survivability of enteric bacteria in the soil. Previous studies have related better survivability of coliforms to an increase in moisture content (Boyd et al., 1969; Tate, 1978). Chandler and Craven (1978) recorded 99 percent reduction of *E. coli* in a single day in dry soil, whereas reduction was less than 90 percent after three weeks in saturated soil. Chandler and Craven (1980a) also recorded the time for a 90% decrease in population, t_{90}, for *E.coli* at 18 days (20°C) in soil with 30 percent moisture. In soil with 10 percent moisture the t_{90} value was 2.5 days. Another study of Chandler and Craven (1980b) showed that when the dry matter content in a soil-water mixture was increased from 1% to 92% in a soil with field capacity of 76% dry

matter, t_{90} values initially increased with the increase in dry matter content from 1% to 60%, but beyond 60% dry matter content, t_{90} values decreased with increase in dry matter content. These results indicate that an increase in soil particle contents in water generally increases survivability of coliform. However, when dry matter content in a soil-water mixture is very high, compared to the water content in the soil, survivability decreases with an increase in dry matter content, due to desiccation. The general relation between dry matter content and survivability is shown in Figure 2.1.

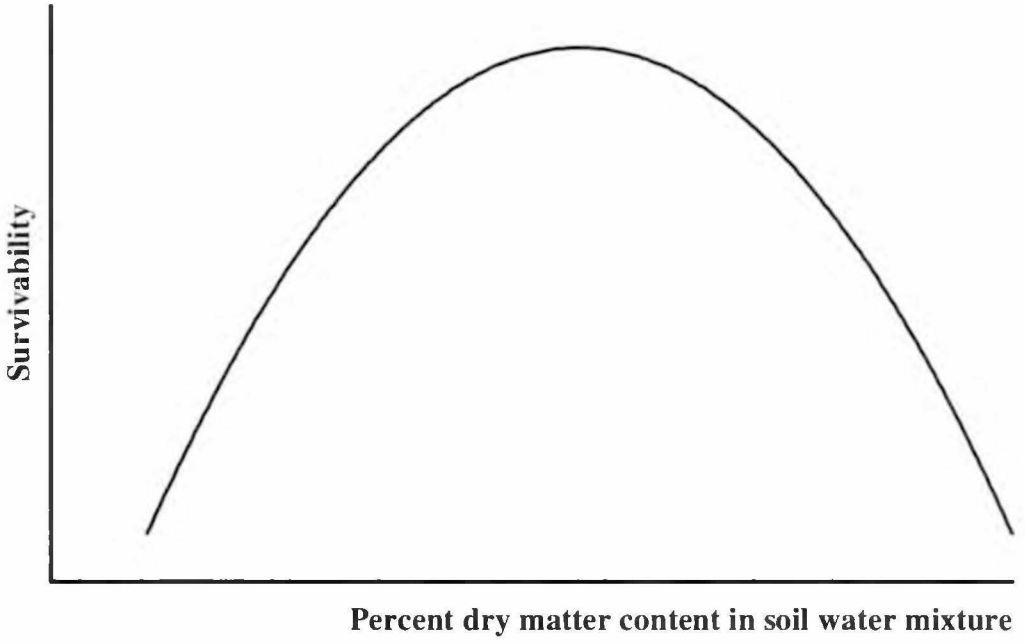

Figure 2.1. General relationship between survivability of coliform and percent dry matter in the soil water mixture.

Soil types with different physical and chemical properties are also associated with the survivability of coliforms. Bushby and Marshall (1977) postulated that fast growing *Rhizobium* species gain desiccation resistance by the presence of clay particles in the soil,

because high water activities of clay lower the internal water content of the adjacent cell. When internal water content of the cell is lowered to the stage where enzyme activities are lowered, survivability of bacteria increases. Howell et al. (1996) also demonstrated that fecal coliform mortality rates were significantly less in the presence of saturated clays in comparison with silt or sand particles. Tate (1978) found extended survival of *E. coli* in fine organic soils in comparison to the mineral laden coarser soil. The larger survival was attributed to the high nutrient content and high water holding capacity of fine soil. In addition to the high nutrient content and high water holding capacity, fine soils provide more protected sites to safeguard microbes against predation (Cools et al., 2001). In contrast to the explanation given by Tate (1978), Mobiru et al. (2000) have shown higher mortality rates of *E. coli* in coarse-loamy soil compared to fine-silty soil, despite greater organic matter content in coarse-loamy soil. These results suggest that available water in the soil is the overriding factor in *E. coli* survival. Irrespective of the temperature and sampling time, Cools et al. (2001) have demonstrated better survivability of *E. coli* in sand with higher organic content than in loamy silt or loamy sand with lesser organic content, suggesting that nutrients are the major factor in survivability of *E. coli*.

All organisms have their own typical cardinal temperature at which they have the highest growth rate. Provided other factors are optimal for cell survival, as temperature increases, reaction rates in the cells and efficiency of cell components increases, leading to better survivability and growth. However, above a certain temperature, some proteins are denatured and efficiency of individual cell components falls sharply. For every cell there is a minimum temperature below which cell growth no longer occurs, an optimum

temperature at which growth is very rapid and maximum temperature above which cell growth ceases. Cardinal temperatures for most of the coliforms lie in the mesophilic range, which is approximately between 8°C and 48°C. Temperatures below the lower limit of cardinal temperatures, including subzero temperatures, do not allow cell growth, but do not necessarily kill the cell (Madigan et al., 2000).

Studies by different researchers have shown that, generally, with an increase in temperature in the non-host environment, survivability of enteric bacteria decreases (Cools et al., 2001; Howell et al., 1996; McFeters and Stuart, 1972; Tanaka et al., 1999). Reddy et al. (1981) estimated that within the temperature range of 5°C to 35°C, the die-off rate doubles with every 10°C rise in temperature.

Survivability of cells in sub freezing temperatures depends more on the surrounding medium than the rate of cooling or thawing. Calcott and MacLeod (1974, 1975) observed that the viability of *E. coli* suspended in distilled water increased with increase in cooling rate up to 6°C/min, after which it decreased. Lowest viability was found at the cooling rate of 90°C/min and above this rate the viability of *E. coli* started increasing. At a very high cooling rate of 950°C/min, viability was above 80%. The viability of *E. coli* was observed to be unaffected by thawing rate for those samples that were cooled at lower rates. For the samples that were cooled above 1000°C/min, a fast rate of thawing provided increased viability. Survivability of *E. coli* suspended in 0.85% saline medium was very low in comparison to the *E. coli* suspended in distilled water. *E. coli* suspended in a protectant medium of 3% glycerol did not show die-off at all when it was frozen at a similar cooling rate. Packer et al. (1965) demonstrated that repeated

freezing and thawing of *E. coli* led to a linear decrease in the log of the number of viable cells as a function of the number of freezing and thawing cycles.

Viability of coliforms will be greatly reduced if they are exposed to solar radiation, but in the absence of solar radiation, viability of coliform will be affected most by temperature, moisture content, and the pH in the medium.

2.3. Die-off kinetics of coliform in a non-host environment

As discussed previously, coliforms face a severe change in the environment once they are excreted by the host. Generally, in non-host environments, coliform populations decrease. Previous studies have assumed that the coliform die-off rate in non-host environments with temperatures above freezing follows first-order kinetics (Avery et al., 2004; Crane et al., 1980; Feachem et al., 1983; Reddy et al., 1981; Sherer et al., 1992; Stoddard et al., 1998). First-order die-off kinetics is also known as Chicks law (Sarikaya and Saatci, 1995). It is also referred to as the exponential decay model. It is described as:

$$\frac{dC}{dt} = k_B C - k_D C = (k_B - k_D)C \qquad (2.1)$$

where, C is equal to concentration of coliform, k_B is the rate coefficient for the rate of coliform division per unit time and k_D is the rate coefficient for coliform death per unit time.

After rearranging and integrating equation (2.1), the relationship between the initial microbial population, C_o, and the microbial population at any time, C_t, is obtained by

$$C_t = C_o \exp^{(k_B - k_D)t}. \quad (2.2)$$

Equations 2.1 and 2.2 are general equations that account for both growth and death of cells. If the die-off rate is greater than the rate of cell division (i.e. $k_D > k_B$), then the net coliform concentration with respect to time decreases. Considering k as the net die-off rate coefficient per unit time for the coliform population as follows:

$$-k = k_B - k_D. \quad (2.3)$$

Equation (2.2) can be written as:

$$C_t = C_o \exp^{-kt}. \quad (2.4)$$

Taking $\log_{10}$ of both sides yields:

$$\log_{10} C_t = \frac{-kt}{2.3} + \log_{10} C_o. \quad (2.5)$$

Rearranging equation (2.5) results in the following expression for k:

$$k = \frac{2.3}{t} \log \frac{C_o}{C_t}. \quad (2.6)$$

Net die-off rates are also expressed as the time for ninety percent of the population to decay, t_{90}, and as the half life of the population, t_{50}. The net die-off rate coefficient, k, and t_{50} and t_{90} values are expressed by the following expressions:

$$k = \frac{2.3 \log 2}{t_{50}}, \text{ and} \quad (2.7)$$

$$k = \frac{2.3}{t_{90}}. \quad (2.8)$$

The first order die-off rate is not always found in all environmental conditions. Davies et al. (1995) tested the survivability of fecal microorganisms in marine and fresh water sediments. Their results showed a curved trend in the data. To test the curvature validity, the model was fitted with the data using quadratic and linear terms as the time variable and tested for goodness of fit. Van Donsel et al. (1967) also noted a slight deviation from the logarithmic trend of die-off of coliform in the study conducted in winter, but nevertheless, the same model was used to describe the survivability character of coliforms.

2.4. Transport of coliform from the soil

It is known that fecal coliforms survive for several months in fecal matter (Bolton et al., 1999; Wang et al., 1996), fecally contaminated soil (Avery et al., 2004; Jiang et al., 2002, Lau and Ingham, 2001) and in a soil and culture mixture (Sjogren, 1995). Surviving coliforms are likely to get transported from their location due to surface runoff, subsurface infiltration of water as well as human and animal activities. Doran and Linn (1979) observed five to ten times more fecal coliforms in rainfall runoff from grazing areas than from non grazed areas. Kistemann (2002) also observed an increase in enteric microbes in tributaries at extreme runoff events. Carney et al. (1975) found large numbers of fecal coliform in the creeks receiving pasture land runoff and runoff from settlement areas.

Enteric bacteria also get transported into the soil by infiltrating water, which typically is a very slow process owing to slow subsurface flow rates and adsorption of a

large number of coliforms onto soil particles. The leaching rate of microorganisms depends on factors like the dry bulk density of the soil, available macro pores (Artz et al., 2005), hydraulic conductivity, and surface slope (Rahe et al., 1978). Leaching rates of *E.coli* were found to decrease with an increase in dry bulk density and were significantly increased with an increase in earthworm burrows (Artz et al., 2005). Rahe et al. (1978) observed significant numbers of *E. coli* present in subsurface water at a distance of 15 m and at a depth less than 50 cm down-slope from the point source just an hour after inoculation in the ground. Transport rates at greater depths were slower than shallow depths. These researchers attributed the relatively rapid transport of the *E. coli* in the upper layer of soil to the presence of macro pores in the soil.

Coliform transport also occurs by anthropogenic activities. Chambers et al. (2005) have showed that vehicles and people coming from open dumpsites in rural Alaska carry coliform along tires and shoes and are likely to contaminate the community.

Chapter Three

Materials and Methods

All experimental procedures and data analysis tools employed in this study are described in this chapter. The first section describes how soil samples were prepared and how physical and chemical tests of the soil were conducted. The second section describes the enumeration procedures for total coliform and the final section describes statistical tools used for analyzing the data obtained.

All media and dilution water were prepared using double deionized water. Soil samples were hydrated with tap water. Dilution water and growth media were prepared and stored in sterilized utensils. Sterilization was accomplished in an autoclave for at least 15 minutes at 120°C. Before and after conducting enumeration experiments, fume hoods where experiments were conducted were sterilized by bleach solution or by 70% isopropyl alcohol solution. Aluminum loops of size 3mm in diameter, used for transferring cultures, were sterilized by flame until the loops glowed. Preparation of different culture media, phosphate buffered dilution water, and enumeration procedures were performed according to the Standard Methods for Examination of Water and Wastewater, Part 9000 (2000). The standard total coliform fermentation technique with multiple tube (five tubes) test was used for total coliform enumeration in each sample. Three replicate of soil samples were tested for each experiment.

3.1. Soil Sample Preparation and soil characterization

Soil used in the study was collected from a field in Fairbanks and air dried for several days. The soil was pulverized in a mortar using a pestle with rubber covered grinding face in order to break the clumps of the soil, but avoid breaking down individual soil particles. Total organic matter in the soil was found by incinerating a pulverized sample at a temperature of 550°C for an hour. Soil pH was measured according to a method presented by Mc Lean (1992). Five grams of air dry soil was mixed with 5 ml of water at high speed with a vortex mixer for at least 5 seconds. After allowing the soil to settle down for 10 minutes, pH was measured using a pH meter with a glass electrode (VWR Scientific, model 8015).

Conventional soil mechanics tests were conducted on the soil used in this research to characterize the soil based on particle size distribution. These tests consisted of: specific gravity, sieve analysis, and hydrometer analysis. These tests were conducted according to test procedures outlined by the American Society for Testing and Materials (ASTM) (1999).

The samples used in this study were prepared manually by mixing non-sterile soil, tap water, and dog fecal matter. Three sets of samples having moisture contents of 24%, 37%, and 49% with respect to dry mass of the soil were made. Samples with 24% and 37% moisture contained 5% of dog fecal matter with respect to total dry mass, whereas the sample with 49% moisture contained 3% of dog fecal matter. Soil moisture was determined by drying a known mass of each sample in an oven at 110°C for 24 hours. Prepared soil samples were placed in sterile Petri dishes and sealed with Parafilm to

avoid loss of moisture from the samples. Samples containing 24% moisture formed small loose clods whereas samples containing 37% and 49% moisture content adhered to the Petri dishes. After enumerating initial MPN of the total coliform, samples from each set were placed in environmental chambers, each at different controlled temperatures. Petri dishes containing samples from each set were also exposed to ambient temperature by placing the samples in a secured container in the open environment. Constant temperatures were 20°C, -5°C, -15°C, -20°C and -28°C. The uncontrolled temperature of the samples was measured using a data logger set to an interval of 30 minutes for recording. Total coliform population in the soil samples was investigated at different time intervals.

3.2. Total Coliform enumeration

For enumeration of the coliform population, 10 g of soil from each sample were serially diluted in 10-fold steps in dilution water. In the first dilution, soil clumps were mixed with a magnetic stirrer for three minutes. Mixing in further dilutions was done by manual shaking. For presumptive enumeration, five culture tubes containing 10.0 ml of lauryl tryptose broth (LTB) were inoculated with 1.0 ml of dilution water and then incubated at 35°C for 24 to 48 hours to observe gas production due to fermentation activities by total coliform. For confirmation of the presence of total coliforms, a 3 mm size loopful of cultures from positive presumptive growth tubes were inoculated into fermentation tubes containing 10.0 ml of brilliant green lactose bile broth and were incubated at 35°C for 24 to 48 hours to observe fermentation. To provide a quality

control on tests for the presence of coliform bacteria, McConkey agar plates were inoculated with cultures from at least 10% of the tubes confirming positive results and then incubated for 14 to 18 hours. Cultures were then transferred from McConkey agar to nutrient agar and into the secondary LTB broth tubes. Inoculated nutrient agar mediums were incubated for 14 to 18 hours at 35°C, whereas LTB broth tubes were incubated for 24 to 48 hours. Cultures from nutrient agar medium were transferred onto glass slides for gram staining to observe the presence of gram negative cells. Samples showing presence of gram negative cells and positive gas fermentation activities confirmed fully the presence of total coliform in the soil. Data obtained were compared with Table 9221-IV of Standard Methods for the Examination of Water and Wastewater (2000) to approximate the total coliform concentration in the soil samples. Since samples with different moisture content had different initial numbers of total coliform, data were normalized with respect to the initial readings. Normalization was done by using following formula:

$$N = \frac{C_t}{C_o},$$

Where, N is the normalized value of the coliform population, C_t is the coliform population in the sample at time t, and C_o is equal to initial coliform population in the sample.

3.3. Statistical analysis

The first-order kinetics model of die-off of coliform bacteria in the non-host environment, as explained in Chapter Two, is used to analyze the results. Simple linear regression is used for statistical analysis. Goodness of fit of the data to the linear model was evaluated by calculating the coefficient of determination and by using software (Minitab version 14) for a lack of fitness test of the linear model. Specific die-off rate coefficients for total coliforms in different soil samples were obtained from the slope of the graph by plotting the mean log of coliform concentration verses time in days. The 95% confidence intervals for the slope and regression line were calculated to find the variability of approximated die-off rate coefficients. In order to compare die-off rates of the coliforms under different experimental conditions, slopes of the regression models were compared at the 95% confidence interval. If slopes of different regression models were found to be not significantly different from each other at the 95% confidence interval, then the survivability of the total coliforms was also considered not significantly different among the models compared.

Chapter Four

Results and discussion

In this chapter, the results obtained from all preliminary studies of soil and coliform survivability, conducted using the methods detailed in the previous chapter, are discussed. Results were analyzed critically and relevant explanations and suggestions are discussed in detail.

4.1. Soil characteristics

Soil tests were conducted in order to identify different physical and chemical properties of the test soil. The particle size distribution of the test soil is shown in Figure 4.1. The specific gravity of the soil was found to be 2.76. According to the United States Department of Agriculture (USDA) soil classification method, soil particles passing through a 2 mm sieve and retained on a 0.05 mm sieve are considered to be sand. Silt is considered to be that soil passing through a 0.05 mm sieve and retained on a 0.075 mm sieve. Clay is considered to be that fraction of soil finer than 0.075 mm. It was found that almost 100% of the soil particles were finer than 2 mm, 72% of the soil particles were finer than 0.05 mm and 11% were finer than 0.075 mm, which means the soil sample contained 28% sand, 61% silt and 11% clay. Thus, the overall textural classification of the soil according to the USDA classification was silt loam (Peck et al., 1972). Silt loam is a typical soil in the greater Fairbanks region (Reiger et al., 1963) and is known as Fairbanks silt. Hydrometer analysis and sieve analysis data used for classifying soil are listed in Appendix A. The dry unit weight of soil was found to be 1.36 g/ml and porosity

of the soil was approximately 0.5. Volumetric water content in the soil sample with 24%, 37%, and 49% moisture contents on a mass basis were 33%, 50% and 67%, respectively. The soil sample with 37% moisture on a mass basis was very close to saturation (98%), whereas the sample with 49% moisture on a mass basis was oversaturated (131%). Both of these samples correspond to very wet soils that are commonly encountered in the Arctic due to poor drainage brought about by permafrost. The soil sample with 24% moisture had a saturation of 65%. Throughout this chapter water content on a soil will be discussed in the mass basis.

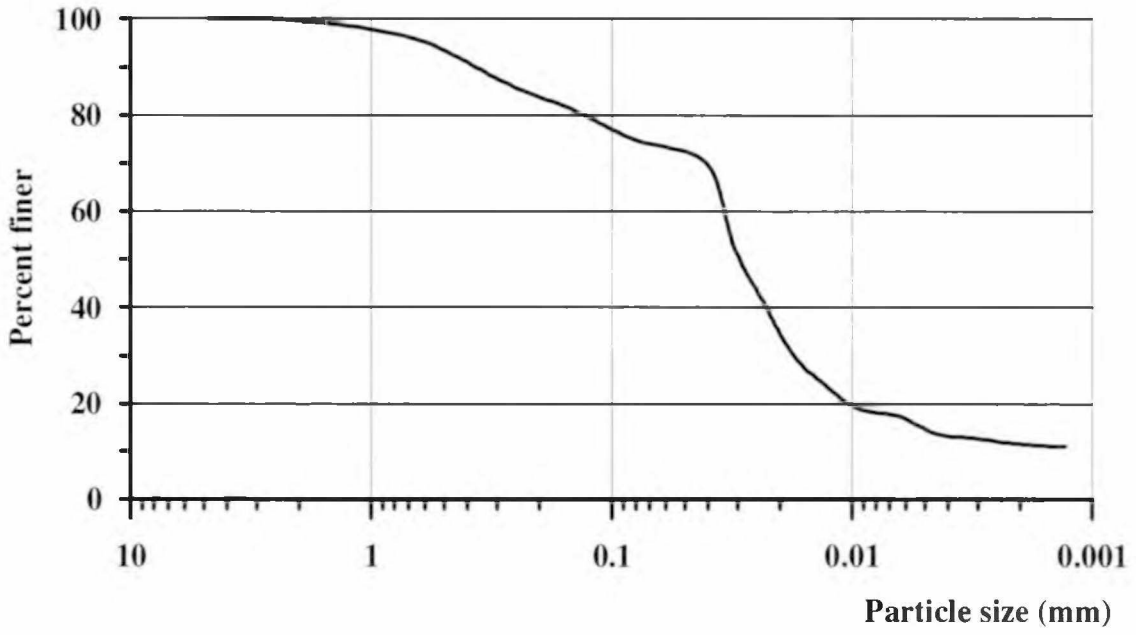

Figure 4.1: Particle size distribution of the soil sample.

The organic matter content in the test soil without any fecal matter was found to be 2.11% of dry soil. The test soil had a pH of 6.44 before mixing with fecal matter. Table 1 shows the pH of combined soil and fecal matter at the different moisture contents after 200 days exposure to the different selected temperatures. The pH of the soil samples

were within the range of 7.4 to 8.6, which is suitable for coliform survivability (McFeters and Stuart, 1972).

Table 1: pH in the soil samples with different moisture contents placed at different temperatures.

Soil sample moistures	Temperature (°C)	pH			Average pH
24%	Room	7.20	7.44	7.57	7.4
	-5	8.56	8.62	8.64	8.6
	-15	8.40	8.38	8.43	8.4
	-20	8.48	8.53	8.56	8.5
	-28	8.33	8.39	8.43	8.4
37%	Room	7.57	7.66	7.67	7.6
	-5	8.54	8.54	8.47	8.5
	-15	8.32	8.40	8.30	8.3
	-20	8.53	8.52	8.18	8.4
	-28	8.06	8.00	8.08	8.0
49%	Room	7.74	7.74	7.73	7.7
	-5	8.02	8.02	7.95	8.0
	-15	7.97	7.84	7.85	7.9
	-20	7.70	7.81	7.93	7.8
	-28	7.72	7.81	7.78	7.8

4.2. Total coliform survivability under constant temperature conditions

Linear modeling of the logarithm of survivability of total coliforms in a non-host environment with respect to time is discussed in Chapter Two. Given the success with linear modeling of coliform survivability (Reddy et al., 1981), linear modeling was used

to describe the general trends in logarithm of coliform survivability with time and determine the die-off rates. Most probable numbers of total coliform obtained in this study, with time in the soil and at each moisture content and temperature, are presented in Figures 4.2 through 4.6. These figures also show best fit linear regression lines as well as confidence intervals on the data points and the 95% confidence interval about the regression lines.

Figure 4.2 shows the relationship between $\log_{10}$ MPN and time at room temperature at three different moisture contents. Change in MPN with time was found to be high at this temperature when compared to that at subzero temperatures (Figures 4.3 through 4.6). Average log MPN values shown in Figure 4.2 for all three moisture contents appear to follow a cyclic trend about the best fit lines. The reason for this apparent trend is unknown. At the lower moisture contents, the 95% confidence intervals about the regression lines are wider compared to those for data obtained for soil with 49% moisture (Figure 4.2 (c)). Wide confidence intervals about the regression lines could have been the result of large variation in replicate samplings for some data, or because of a larger distance between some data points and the best fit line. Wide confidence intervals about data points result from large variation in replicate samplings, which could have occurred due to experimental errors.

As shown in Figure 4.3, at a temperature of -5°C for each moisture contents, coliform survivability appears to be greatest in the soil with 24% moisture content. This trend was replicated for the other subzero temperatures investigated in this study (Figures 4.4 through 4.6). Data points obtained at -5°C for 24% and 37% moisture contents were

close to the regression lines, but at 49% moisture, the 95% confidence interval of one of the data points lay outside the 95% confidence interval of the regression line. This outlier could be the reason for the wide 95% confidence interval about the regression line at the 49% moisture content. Similar results were found for two other data sets obtained in this study (-15°C, 37% moisture content and 28°C, 24% moisture content)

A basic assumption for fitting any model to a set of data is that the resulting model used for the study is valid. Estimation of the model parameters requires the assumptions that errors are random variables with a mean zero and are normally distributed. Adequacy of a linear regression model is commonly judged by the coefficient of determination (R^2), which can be determined by the following equation (Montgomery and Runger, 2003):

$$R^2 = \frac{SS_R}{SS_T} = 1 - \frac{SS_E}{SS_T} \qquad (4.1)$$

where SS_R is the regression sum of squares, SS_E is the error sum of square and SS_T is the total sum of square.

Considering Y as a dependent variable ($\log_{10}$ MPN) and X as an independent variable (time), SS_R is the sum of squares of the difference between fitted Y values in the model and the average of observed Y values. SS_R can be interpreted as a measure of how much variation in Y is left unexplained by the model. SS_T is the sum of squares of the difference between observed Y values and the average of observed Y values and is a quantitative measure of the total amount of variation in observed Y. Thus, the R^2 value

refers to the amount of variability in the data accounted for by the regression model. A perfect linear relationship corresponds to an R^2 value of one.

As shown in Table 4.2, high R^2 values were obtained for the test conducted at room temperature. A generally decreasing trend of R^2 values was observed with a decrease in temperature. Since the observed Y values at subzero temperatures did not vary substantially with time in the study, values of SS_R were very small in comparison to SS_T values. Thus, at subzero temperatures, R^2 values were likely to be very low relative to tests conducted at room temperature. High R^2 values are possible to obtain if observed Y values are very close to the fitted linear regression model, even if the fitted model is not correct. So it is necessary to conduct a statistical lack of fit test on the model to verify the accuracy of the model.

If a data set is assumed to be explained by a linear model and it is not linear, an assumption of linearity will introduce a bias into an estimate of error variance (Belz, 1973). To test the lack of fit of the model, two hypotheses are considered. The first hypothesis was that the regression model is linear and the second hypothesis, which is an alternate hypothesis, was that the regression model is not linear. Error mean square (MS_E), which is calculated by dividing SS_E by the degrees of freedom, is an estimate of variance based on the assumption that the linear model is correct. If there is more than one Y observation at different X values then the sample variance computed at each X value can be pooled to estimate the variance which does not depend upon the linear model being correct. By comparing two estimates of variance for two different values, the appropriateness of the linear model can be tested. If there is more than one observation at

different X values, then SS_E can be partitioned into pure error mean squares (SS_{PE}) and lack of fit sum of squares (SS_{LF}) (Neter and Wasserman, 1974). Their corresponding mean squares are pure error mean square (MS_{PE}) and lack of fit mean square (MS_{LF}). MS_{PE} is an unbiased estimator of error variance. The ratio of MS_{LF} and MS_{PE} gives the F value for lack of fit, which is compared with standard F distribution values at the desired percentile of confidence. If the calculated F value exceeds the standard F distribution at the desired confidence level, then the hypothesis of the regression model being linear is rejected (Neter and Wasserman, 1974).

Lack of fit tests for linear models were conducted by using software (Minitab version 14.2). Data obtained from samples with 24% moisture content, placed at -5°C and -15°C, did not indicate lack of fit of the linear model, whereas the same samples placed at room temperature, -20°C and -28°C, indicated some possibility of lack of fit. This could be because of possible lack of fit of the model at outer X values (time) or because of a curvature trend in the data. Data obtained from the samples with 37% moisture, placed at -20°C and at room temperature, also indicated possible lack of fit with the linear model. The soil samples with 49% moisture, placed at -5°C and -20°C, indicated curvature in the trend of data, whereas outer X-values (times) of the same sample placed at -15°C indicated some possibility of lack of fit. Although some sets of samples have evidence of lack of fit in the model, an assumption was made that the data were adequately described by a linear model of $\log_{10}$ MPN versus time, in order to compare the die-off characteristics at different moisture and temperature conditions with respect to time. This model was found to be the best approach to describe the die-off rates. While rate

coefficients and half-lives determined from the linear models that did not fit the data well may not be accurate, at a minimum, use of the linear model allows for an adequate description of the trend in each of the data sets.

The slope obtained from the regression is a mean estimated slope. By considering that error terms in regression models are normally distributed, it is possible to obtain confidence intervals for slopes and Y-intercepts. The width of the confidence interval is a measure of the overall quality of the regression line (Montgomery and Runger, 2003). As there is variability in slopes and Y intercepts, Y values at different X values also have variability. A confidence interval can be constructed on the mean response (i.e. fitted Y values) at a specified value of X. This confidence interval is also called a confidence interval about the regression line (Montgomery and Runger, 2003). By repeating calculations for all different X values, a confidence limit for each corresponding value of the mean response at specified intervals is obtained. A plot of upper and lower confidence intervals of mean response values for different X values are parabolic in nature, as shown in Figures 4.2 to 4.6. The confidence interval for the mean response value is minimal for the observed X value, when it is equal or close to the average X value. This is because confidence intervals about the regression line are a function of the sum of squares of the difference between the observed X values and the average observed X values.

The data used for plotting the graphs of survivability of coliform versus time were: 95% confidence intervals of the total coliform population at different times obtained from the replicate tests, 95% confidence intervals of the slopes of the regression lines, and 95%

confidence intervals of mean response values of MPN at different times due to fitting of data with linear models. These data are provided in Appendix B.

Slopes obtained from $\log_{10}$ MPN versus time graphs were used for calculating specific die-off rate coefficients (k) of total coliform using equation (2.5). Table 4.1 lists these coefficients at different temperature and moisture conditions. The table also lists estimated half lives and the 95% confidence intervals for specific die-off rate coefficients. Slopes were compared statistically by hypothesis testing. The first hypothesis considered was that two slopes were equal, and its alternative hypothesis was that two slopes were not equal. If the 95% confidence intervals of the difference between two slopes captured the zero value, then it was concluded that the slopes considered were equal.

The average specific die-off rate coefficients of total coliform in the soil samples with 24% moisture that were placed at room temperature was relatively high (0.041/day), when compared to soil with the same or greater moisture contents at room temperature and at other temperatures. Specific die-off rate coefficients in the soil with 24% moisture and placed at different subzero temperatures were very low (below 0.007/day).

Soil samples with 37% moisture content also had high average specific die-off rate coefficients at room temperature (0.035/ day) when compared to the same samples placed at subzero temperatures. Specific die-off rate coefficients between -5°C and -15°C, were not significantly different from each other, but the specific die-off rate coefficient at -5°C was different from that at –20°C and -28°C at the 95% confidence interval. The specific die-off rate coefficients at -15°C, -20°C and -28°C were not significantly different from each other at 37% moisture.

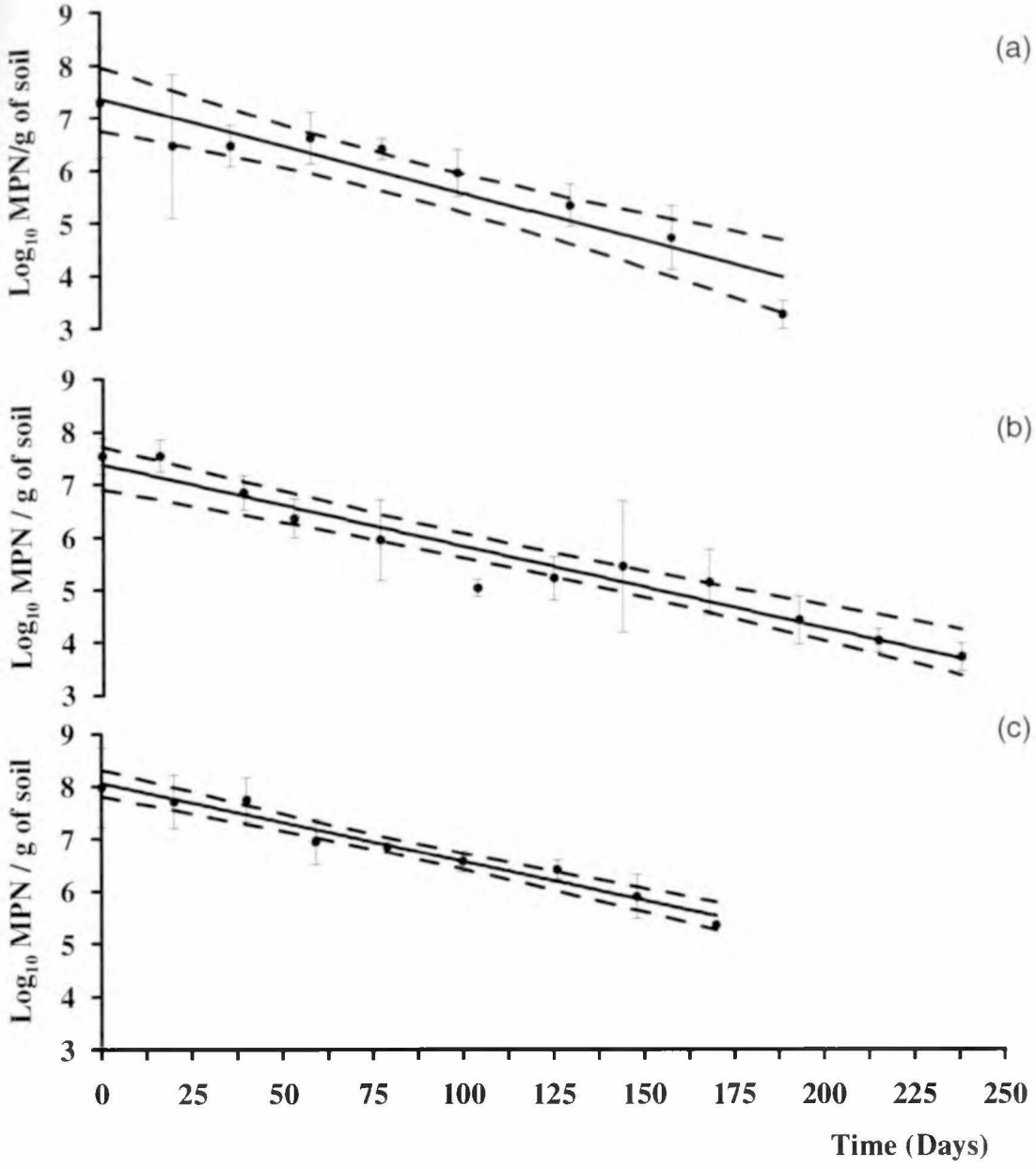

Figure 4.2: Survivability of total coliform in the soil samples at room temperature with (a) 24% moisture, (b) 37% moisture, and (c) 49 % moisture. The 95% confidence intervals for replicate measurements are shown with interval bars. Solid lines represent the best fit linear model and dotted lines represent 95% confidence intervals about the regression line.

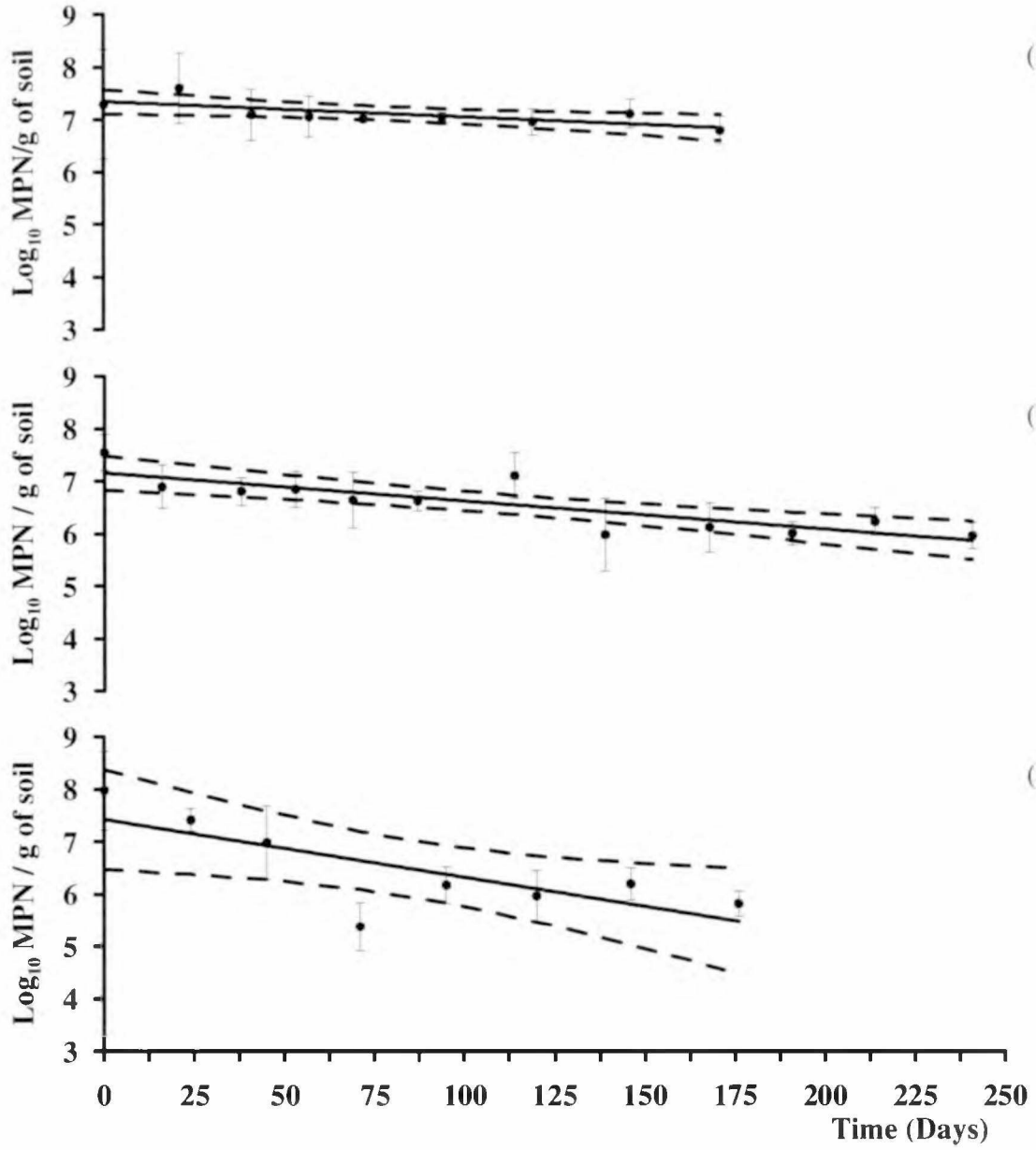

Figure 4.3: Survivability of total coliform in the soil samples at -5°C with (a) 24% moisture, (b) 37% moisture, and (c) 49 % moisture. The 95% confidence intervals for replicate measurements are shown with interval bars. Solid lines represent the best fit linear model and dotted lines represent 95% confidence intervals about the regression line.

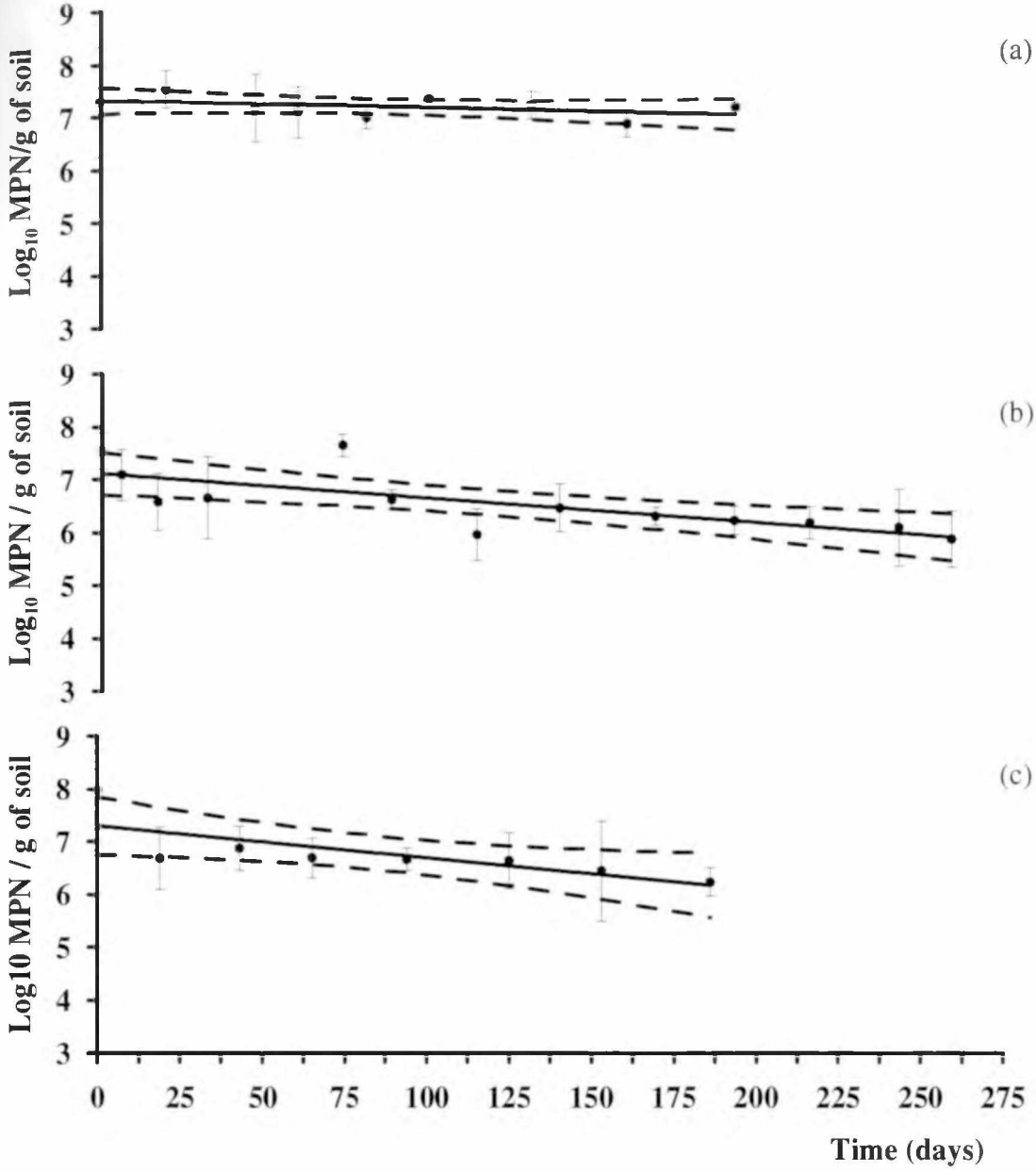

Figure 4.4: Survivability of total coliform in the soil samples at -15°C with (a) 24% moisture, (b) 37% moisture, and (c) 49 % moisture. The 95% confidence intervals for replicate measurements are shown with interval bars. Solid lines represent the best fit linear model and dotted lines represent 95% confidence intervals about the regression line.

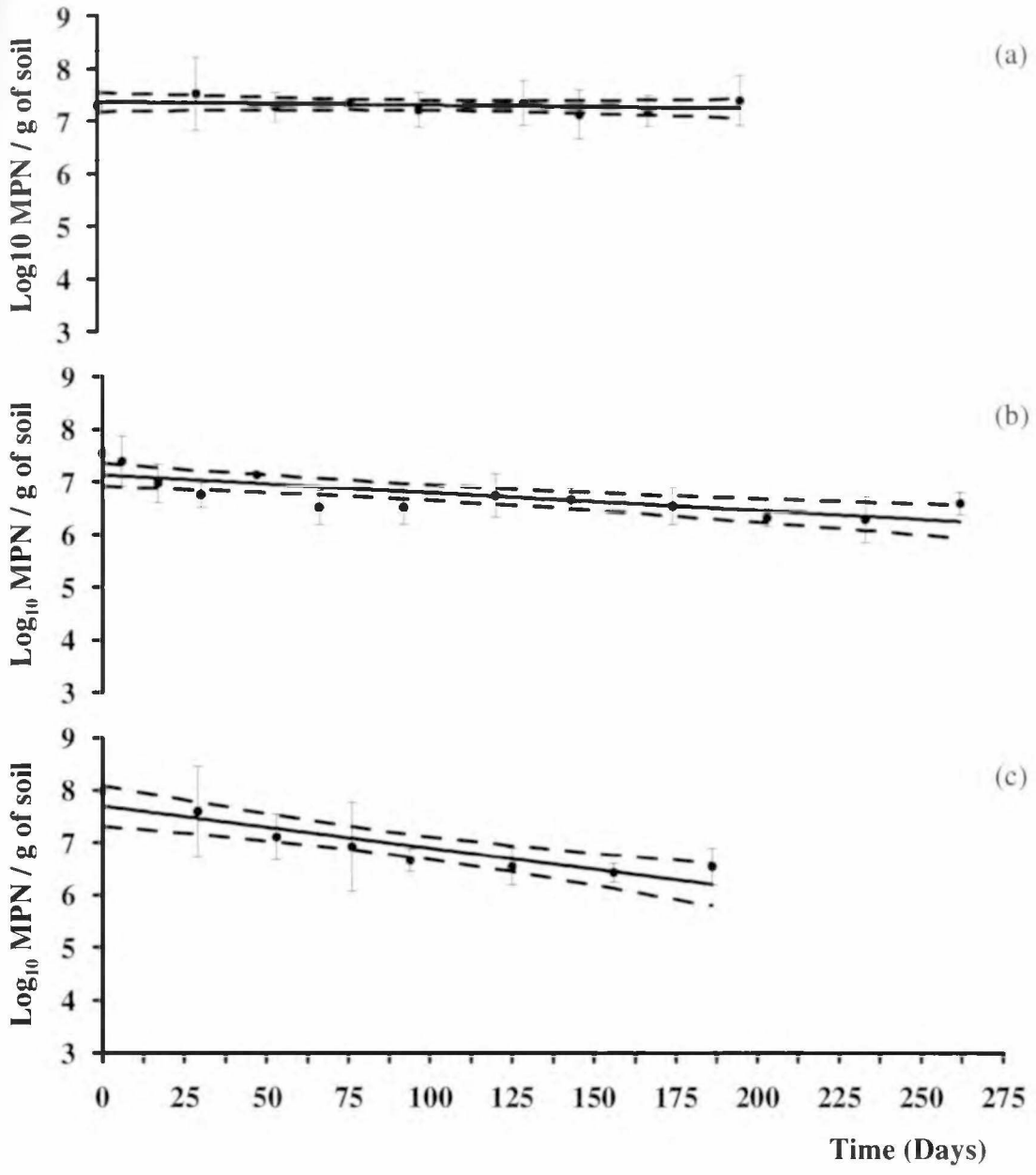

Figure 4.5: Survivability of total coliform in the soil samples at -20°C with (a) 24% moisture, (b) 37% moisture, and (c) 49 % moisture. The 95% confidence intervals for replicate measurements are shown with interval bars. Solid lines represent the best fit linear model and dotted lines represent 95% confidence intervals about the regression line.

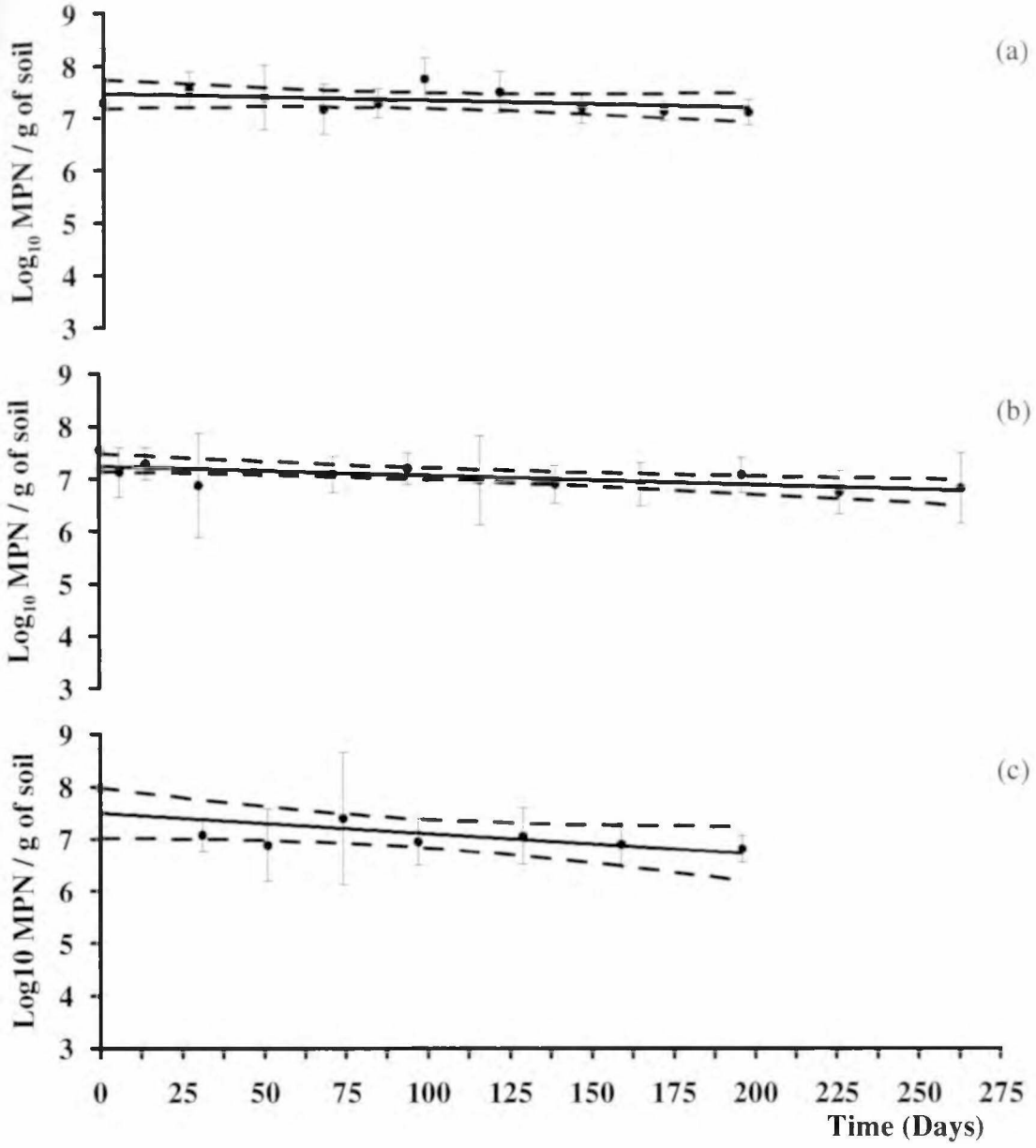

Figure 4.6: Survivability of total coliform in the soil samples at -28°C with (a) 24% moisture, (b) 37% moisture, and (c) 49 % moisture. The 95% confidence intervals for replicate measurements are shown with interval bars. Solid lines represent the best fit linear model and dotted lines represent 95% confidence intervals about the regression line.

Generally, die-off rate coefficients of total coliform in samples with 49% moisture were larger than those of the soil samples with 24% and 37% moisture at the respective temperatures. The specific die-off rate in the soil with 49% moisture content at room temperature was not significantly different from die-off rate at -5°C, whereas specific die-off rates at other subzero temperatures were significantly different from that at room temperature. Die-off rates at -5°C, -15°C, -20°C were not significantly different from each other, but the die-off rate at-5°C differs from the die-off rate at -28°C.

At room temperature the die-off rates were not significantly different from each other. At -5°C, the die-off rates at 24% and 37% moisture were not significantly different, but the die-off rate at 49% moisture was higher than the die-off rate at 24% and 37% moisture. At -15°C, -20°C and -28°C, the die-off rates at 37% and 49% moisture were not significantly different, but the die-off rate at 24% was lower than the die-off rate at 37% and 49% moisture.

Half-lives of total coliform, which are also shown in Table 4.2, were calculated from equation (2.7). These values vary inversely with the specific die-off rate coefficient. As shown in Table 4.2, half lives determined in this study varied from 16 days to 301 days as a function of the different test conditions. Short half-lives were observed at warm and long half-lives were observed at cold controlled temperatures. At subzero temperatures, half lives were found to decrease with an increase in moisture content.

Table 4.2. Die-off rates of total coliform and coefficient of determination of the regression model at different temperatures and moisture contents.

Soil sample moisture	Temperature (°C)	R^2 (%)	Lack of fit test for linear model	The 95% Confidence interval of specific die-off rate coefficients, k, (per day)		Specific die-off rate coefficient, k, (per day)	Half-life of total coliform (days)
24%	Room	83.3	N/F	0.048	0.035	0.041	16.7
	-5	27.7	F	0.012	0.002	0.007	100.3
	-15	9.8	F	0.007	0.000	0.002	301.0
	-20	2.1	N/F	0.005	0.000	0.002	301.0
	-28	8.2	N/F	0.007	0.000	0.002	301.0
37%	Room	89.0	N/F	0.037	0.030	0.035	20.1
	-5	63.8	F	0.016	0.009	0.012	60.2
	-15	49.7	F	0.014	0.007	0.012	60.2
	-20	54.6	N/F	0.009	0.005	0.007	100.3
	-28	31.1	F	0.007	0.002	0.005	150.5
49%	Room	92.1	F	0.039	0.030	0.035	20.1
	-5	54.8	N/F	0.035	0.016	0.025	27.4
	-15	47.1	N/F	0.021	0.007	0.014	50.2
	-20	69.4	N/F	0.023	0.014	0.018	37.6
	-28	29.3	F	0.016	0.002	0.009	75.3

Note: N/F indicates the linear model does not fit the data and F indicates the data fit the linear model

In summary, the resulting general trends found in these controlled temperature coliform survivability studies were: (1) At all moisture contents, the die-off rates increased with increasing temperature, and (2) die-off rates increased with increasing

moisture content. While no other study has reported the survivability of coliforms in frozen soil, results obtained in this study were compared to similar published survivability studies conducted in soils kept at temperatures above 0°C.

McFetters and Stuart (1972) studied the survivability of *E. coli* with respect to temperature. These researchers varied temperature from 5°C to 25°C and found the die-off rate for *E. coli* to be nine times greater at 25°C compared to 5°C. This result is consistent with the trend of increasing die-off rates with increasing temperature found in the current study.

Crane et al. (1980) studied the die-off pattern of fecal coliform in the lab at a controlled temperature (25.5±2°C) and relative humidity of air (70±5 %). Their experiment was conducted for a 30 day period. The coefficient of determination of the linear model of $\log_{10}$ MPN versus time and the specific die-off rate coefficient as low as 10% and 0.031 per day, respectively. Assuming no outside factors such as ultraviolet radiation exposure were influencing the degradation of coliform in the soil sample, the low values of coefficient of determination of the model and specific die-off rate coefficients were comparable to the values obtained in this study.

Reddy et al. (1981) provided a summary of first order die-off rate constants for organisms found in soils. Die-off coefficients determined in the field studies listed in this summary were several orders of magnitude greater than the die-off rates determined in the controlled studies conducted by Crane et al. (1980) and die-off rates determined here and shown in Table 4.2. The differences between the field studies presented in Reddy et al. (1981) and Crane et al. (1980), as well as the results shown in Table 4.2, indicate that

other environmental factors may be influencing the die-off of coliforms, such as ultraviolet oxidation.

Most of the survivability studies for fecal coliform listed by Reddy et al. (1981) were conducted either in the water environment or in the exposed land, where many factors such as nutrient content, sunlight and moisture were not monitored. These factors play a prominent role in microbial survival. The silt loam used in this study already contained 2.11% of organic matter before mixing in 3-5% of fecal matter. Thus, the amount of carbon content can be considered to be relatively high. Though samples contained high organic matter, coliforms in the samples placed in subzero temperatures were not likely to utilize these nutrients, as they were likely to be metabolically inactive. Thus, nutrient availability as a factor in survivability could be considered only at the temperature at which coliforms are metabolically active, i.e., room temperature.

Test samples used in this study were protected against the exposure to sunlight and moisture loss. Sarikaya and Saatci (1995) demonstrated that survival of coliforms in water not exposed to solar radiation was several times greater than in water exposed to the solar radiation. Van Donsel et al. (1967) studied survivability of fecal coliforms during different seasons in a shaded field and a field exposed to the sunlight. The die-off rate was found to be highest in summer in the exposed field and lowest in winter in the shaded field. The results from these two studies indicated that solar radiation could, in part, account for the greater die-off rates in the studies summarized in Reddy et al. (1981). At cooler temperatures Van Donsel et al. (1967) also found the die-off pattern slightly varying from an ideal logarithmic curve. However, the logarithmic pattern was found to

be the best approach to describe the die-off rates. The study of Van Donsel et al. (1967) and this study are comparable in terms of temperature and it can be concluded that in colder temperatures survivability of coliform is higher than at warmer temperatures but very high die-off rates in the study of Van Donsel et al. (1967) can be related to the field site where other environmental factors were not controlled.

The relatively high survivability of total coliforms at low temperatures can best be explained by examining the physiological changes that occur as cells are cooled. As discussed in Chapter Two, the viability of coliforms increases with a decrease in temperature. At subzero temperatures survivability depends upon the surrounding medium and the rate of cooling and thawing. Intracellular or extracellular ice formation and increase in solute concentration are considered to be factors leading to cell death (Mazur, 1965). The survival, following the low temperature exposure, would be maximal when the formation of intracellular ice is minimal. Mazur (1963) describes that when a cell is cooled in an aqueous medium and temperature in the cell protoplasm has just dropped below the freezing point, intracellular water will not freeze immediately, but will become supercooled, resulting in a higher vapor pressure inside the cell in comparison to the external medium. If no water or solutes move in or out of the cell, the ratio of the internal and external vapor pressures would increase progressively with a decrease in temperature. Since the cell membrane is permeable, water moves out of the cell in order to neutralize the pressure difference, leading to an increase in concentration of the protoplasm. If the cooling rate is slow ($<10^{o}$C/min), enough water will leave the cell to

eliminate pressure differences and keep the protoplasm at its freezing point without allowing the formation of ice crystals inside the cell.

Dumont et al. (2004) also suggested that at a slow freezing rate, the rate of heat liberated by water flowing out of the cell is not sufficient for forming ice crystals. Thus crystallization does not occur. Whereas, at intermediate cooling rates (100°C/min to 1000°C/min), ice crystals form. The freezing rate will induce intracellular crystallization during the osmotic exit of water, leading to cell injury. At very high cooling rates, heat transfer is so rapid that intracellular crystallization occurs before any water flows out of the cell. Cryo-preservation during heat and mass transfer from the cell at low temperature can be influenced by the surface to volume ratio of the cell, cell permeability properties, and the presence of cell walls. A high surface to volume ratio, such as those of small or rod shaped cells (such as *E. coli*), leads to faster transfer of heat and mass from the cell, aiding cell survival even at a higher cooling rate. Permeability properties of the cell membranes also control the heat and mass transfer in and out of the cell in response to the freezing rate. Souzu (1980) discusses the cell membranes and the cell wall damage during the freeze thaw cycles. The presence of a strong cell wall gives mechanical support against the damage due to intracellular crystallization.

As detailed in Chapter Two, survivability of coliforms in soil at temperatures above 0°C increases with the moisture content (Boyd et al., 1969; Chandler and Craven, 1978; Chandler and Craven, 1980a; Tate, 1978). Results found in the current study indicate an inverse relation between moisture content and coliform survivability, which is

counter to the results found in temperate soils. This trend was repeated in the uncontrolled temperature studies and will be discussed further later in the chapter.

4.3. Total coliform survivability under uncontrolled temperature conditions

Most probable numbers of total coliforms in the soil at different moisture contents, placed at uncontrolled temperatures are presented in Figure 4.7. The figure shows the best fit linear models for $\log_{10}$ MPN of survivability of coliform as a function of time, 95% confidence intervals on the data points, and a 95% confidence interval about the regression lines. It also shows temperature variations during the test period.

At 24% moisture, sample points were close to the best fit line and they were randomly distributed about the model. At 37% moisture, the 95% confidence intervals of two of the data points did not fall within the 95% confidence interval of mean response values of the best fit line. The 95% confidence interval of one of the data points at 49% moisture also did not fall within the 95% confidence interval of the mean response value about the regression line. Data points at 37% and 49% moisture appear to show some curvature and a lack of fit test also indicated curvature in the model. The data were assumed to be $\log_{10}$ linear, as was assumed for the controlled temperature data, to allow comparison of these data sets with each other. The R^2 values at uncontrolled temperatures, as shown in Table 4.3, were relatively higher than the values at controlled subzero temperatures, which indicated that a relatively higher fraction of the variability in the data was accounted for by the linear model.

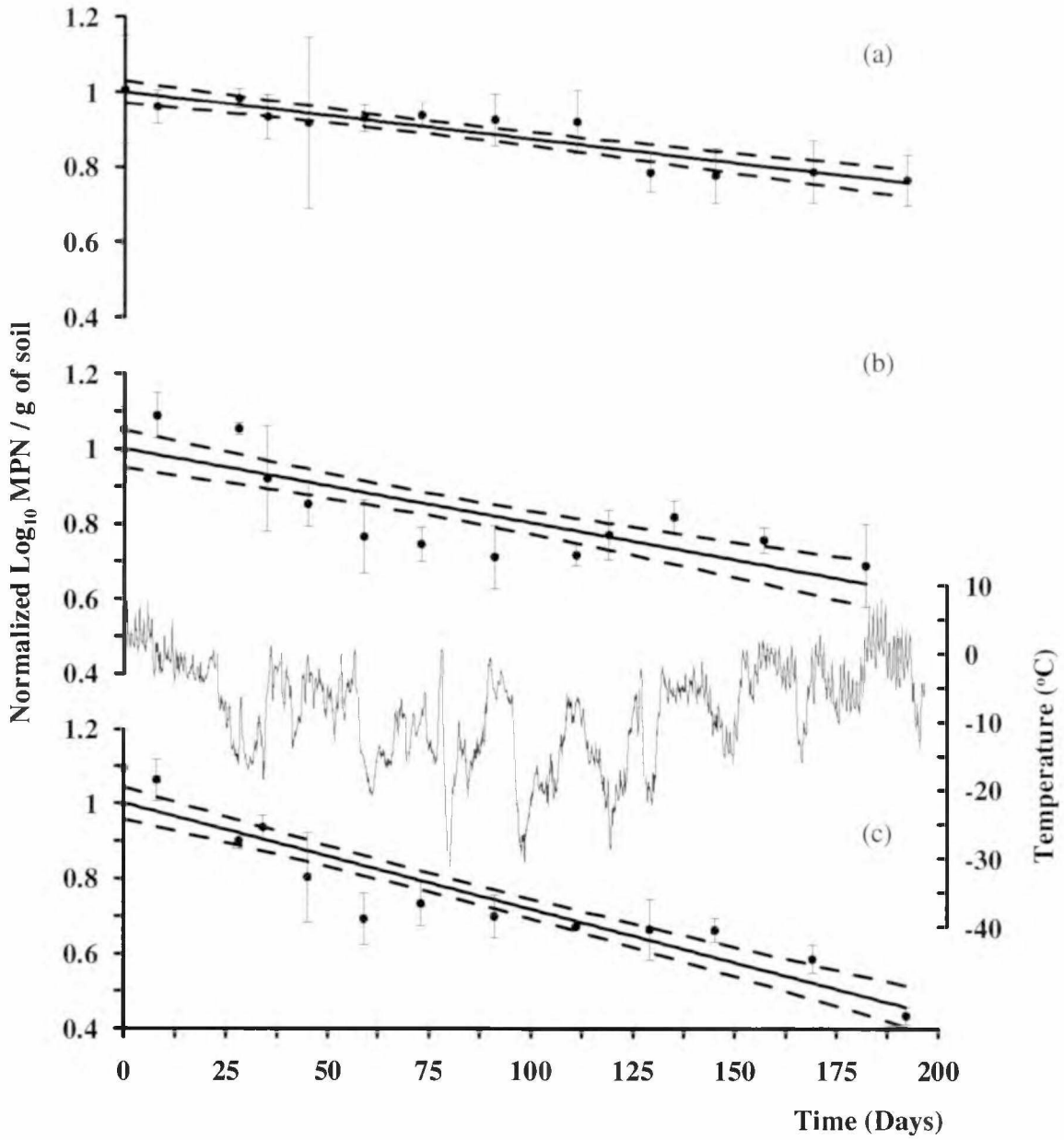

Figure 4.7: Survivability of total coliform in the soil at uncontrolled temperature with (a) 24% moisture, (b) 37% moisture, and (c) 49 % moisture. The 95% confidence intervals for replicate measurements are shown with interval bars. Solid lines represent the best fit linear model and dotted lines represent 95% confidence intervals about the regression line. The irregular line represents temperature variation.

Table 4.3. Die-off rates of total coliform and coefficient of determination of the regression model for incubations at uncontrolled temperatures and different moisture contents.

Soil sample moistures	Temperature (°C)	R^2 (%)	Lack of fit test	The 95% Confidence interval of specific die-off rate coefficients, k, (per day)		Specific die-off rate coefficient, k, (per day)	Half life of total coliform (days)
24%	Uncontrolled	67.4	Fit	0.025	0.016	0.021	33.4
37%	Uncontrolled	61.4	Not fit	0.035	0.021	0.028	25.1
49%	Uncontrolled	83.1	Not fit	0.055	0.039	0.046	15.1

At uncontrolled temperatures, variations in temperature of the samples were due to the variation in temperature in the atmosphere which can be a relatively slow process. Packer et al. (1965) observed a linear decrease in the logarithm of number of the surviving cells of *E. coli* as a function of the number of freeze thaw cycles. The tests were conducted by placing cells in basal salts medium at -78°C for 20 minutes and then at 11°C for 20 minutes, sixteen times. They concluded that the time for which cells were placed at different freezing and thawing temperatures was not critical, because when cells were stored at -78°C for 10 minutes to 12 hours and at 11°C for 20 minutes to 1week, linear models obtained from different experiments did not differ from each other. The temperature in our study first dropped below freezing on 10/9/2004. A temperature data logger was set to record temperatures at an interval of 30 minutes, so temperature fluctuations within 30 minutes were not recorded. In the month of October, temperature

fluctuated about the 0°C mark twelve times and the data logger recorded below freezing temperatures for most of the test period. In November, the temperature was above 0°C once and in December it was above 0°C twice. The extreme temperature of -30°C was encountered in the last week of December. In January and February of 2005, the temperature remained below 0°C all the time. In the first week of March, the temperature reached above 0°C, and it fluctuated about 0°C thirteen times within that month. In April, the temperature fluctuated about 0°C more than eleven times. A possible linear decrease in the logarithm of surviving coliform with repeated freeze thaw cycles was not considered in this study, because it was not known exactly how many times the temperature fluctuated about 0°C, but repeated freeze thaw cycles could have been a major factor for the greater death rate of coliform in uncontrolled temperatures in comparison to the controlled subzero temperatures.

Comparing die-off rates of total coliforms at uncontrolled temperatures with die-off rates at controlled temperatures at similar moisture levels, it was found that at 24% moisture, the die-off rate at uncontrolled temperature was less than the die-off rate at room temperature, but higher than the die-off rates at subzero temperatures. At 37% and 49% moisture contents, die-off rates at uncontrolled temperatures were not significantly different from the die-off rates at room temperature, but die-off rates at controlled subzero temperatures were significantly less than those at uncontrolled temperatures at the respective moisture contents. Half-lives of total coliforms as shown in Table 4.3 varied from 15.1 to 33.4 days. At 24% and 37% moistures, half-lives at room temperature were lower than those at the uncontrolled temperatures at a given moisture, but half-lives

at controlled subzero temperatures were higher than those at uncontrolled temperatures. The half life at uncontrolled temperatures at 49% moisture was lower than the half-lives at all controlled sub-freezing temperatures.

The average specific die-off rate coefficients obtained from samples with 24%, 37% and 49% moisture and placed at uncontrolled temperature were found to be 0.021/day, 0.028/day and 0.048/day, respectively (shown in Table 4.3). As in the controlled temperature tests, these values increased with increasing moisture content, but it was found that at the 95% confidence interval, specific die-off rate coefficients for soils with 24% and 37% moisture were not significantly different from each other. The specific die-off rate coefficient of coliforms in soil samples with 49% moisture was significantly greater than that of 24% and 37% moisture at 95% confidence intervals.

The existence of unfrozen water in frozen mineral and organic soil is well established (Anderson, 1967; Williams, 1963; Williams, 1964). Water in the liquid phase in frozen soil is confined to small pore spaces and particle surfaces. As such the amount of unfrozen water is independent of the total moisture content in the soil water-ice mixture (Williams and Smith, 1989). The amount of water in the liquid phase is dependent on the composition of the soil and the temperature. Fine grains and small pores in the soil generally have a relatively greater amount of unfrozen water content. A decrease in temperature reduces the unfrozen water content.

Two possible factors may have influenced the greater survivability of coliforms. As discussed in Chapter Two, Chandler and Craven (1980b) showed that an increase in the dry matter content influenced the survivability of coliforms. A greater fraction of

solids, and thus particle surface area, in the soil with 24% moisture content may have provided a greater fraction of unfrozen water in comparison to the soils with greater moisture content prior to freezing (37% and 49%). This may have increased survivability in soils with greater unfrozen water content (lower pre-freezing moisture content).

Yershov (1998) discusses the temperature change with time during supercooling and crystallization of water in fine grained soils. According to this author, the lower the pre-freezing moisture content, the higher the cooling rate in the soil. Figure 4.8 adopted from Yershov (1998) shows the rate of cooling becomes important when considering the survival rate of frozen bacteria. As discussed previously, the greater the rate of cooling, the greater the survivability of bacteria up to approximately 10°C/min (Mackey, 1984). Beyond this freezing rate, the survivability decreases. Figure 4.9 adopted from Mackey (1984) shows the effect of cooling rate on survival of frozen and thawed bacteria. A similar pattern of viability for *E. coli* was also reported by Calcott and MacLeod (1974).

The rate of cooling for the soil tested in this research was found to be relatively slow, owing to the method by which the samples were frozen. Mackey (1984) defines cooling rate as the rate at which the freezing cell cools – not necessarily the rate at which the sample cools. Assuming that survivability is a function of ice formation in the cell, the rate at which the sample cools from 0°C to the point where the latent heat is lost from the sample and ice forms in the soil pore space is the most critical rate. For the different moisture contents in this study, these cooling rates were calculated to be approximately 0.4°C/min for the soil with 24% moisture and 0.03°C/min for the soil with 37% and 49% moisture. Survivability of bacteria at different cooling and thawing rates, as shown by

Mackey (1984) in Figure 4.9, does not include the cooling range below 1°C/minute. Assuming the trend of survivability of coliform below the cooling rate of 1°C/minute follows the trend above this cooling rate, the survivability of coliform at a higher cooling rate would be greater than that at a lower cooling rate. Since soil with 24% moisture cooled faster than the soil with 37% and 49% moisture, this could be the reason for greater coliforms survival in the soil with 24% moisture than in the soil with 37% and 49% moisture.

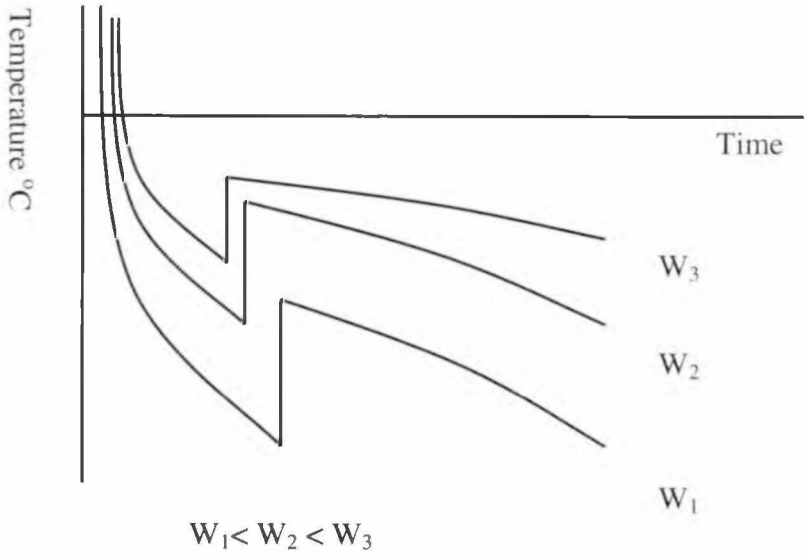

Figure 4.8. Cooling curves of the soil with different moisture contents. W denotes moisture content in the soil. The figure is not to scale and it is adapted from Yershov (1998).

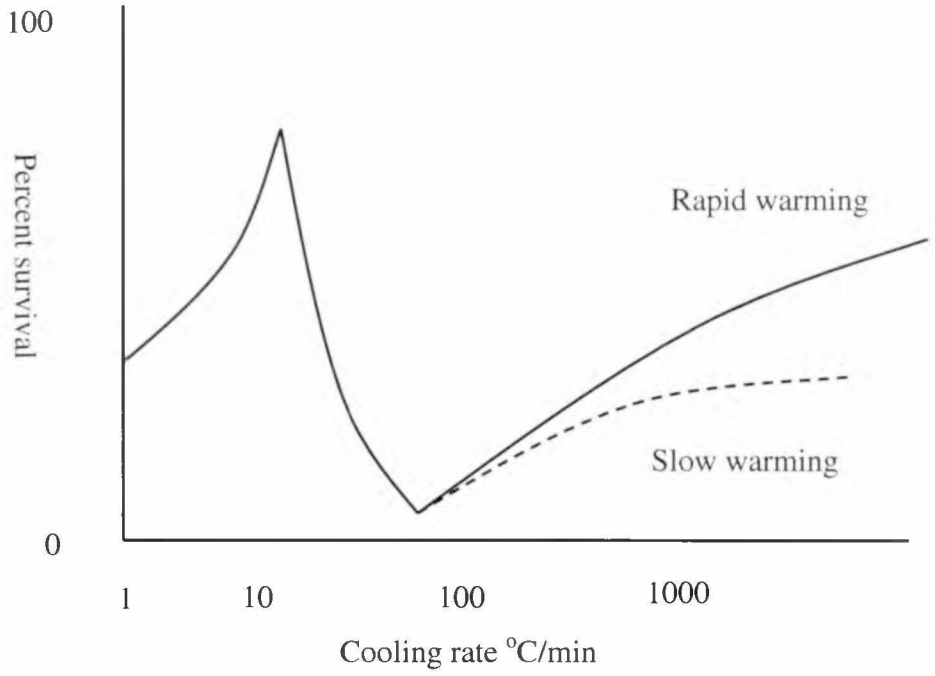

Figure 4.9. Survivability of bacteria at different cooling and thawing rates. The figure is not to scale and it is adapted from Mackey (1984).

Intracellular or extracellular ice formation is one of the factors that causes cell injury while freezing. In slow freezing processes, extracellular water freezes first. The extracellular freezing increases the solute concentration outside the cell. The cell releases water to increase the intracellular concentration, but there is already some unfrozen water around the cell adsorbed on the surfaces of the mineral particles. Thus, effects due to increased concentration of solute around the cell are decreased to some extent by unfrozen water around the mineral surface. Furthermore, Mazur (1966) states that *E. coli* cells are not killed just because of crystallization of the external medium. The plasma membrane of the cell keeps extracellular ice from nucleating intracellular supercooled

water above certain temperatures (Mazur, 1966). If extracellular ice formation is rendered by unfrozen water around mineral particles, then the possibility of intracellular ice formation is also reduced. At 24% moisture, a relatively higher number of cells survived, in comparison to survival at higher moisture content at corresponding subzero temperatures. This could have been because of lowered supercooling temperature in the soil at lower moisture content, as shown by Yershov (1999) in Figure 4.8.

Laboratory studies of survivability of total coliform in the soil have shown that cells exposed to the subzero temperatures survive for several months in controlled and uncontrolled temperatures and constant moisture conditions without exposure to solar radiation. In an open environment, total coliforms may not survive as long as those in the laboratory environment, because of the uncontrolled amount of moisture and exposure to solar radiation. Still, total coliforms are likely to survive for several months.

Fecal matter disposed of in open dumps is likely to support total coliform and other enteric microorganisms throughout the winter and may pollute water sources during spring melting and precipitation runoff. Thus it is necessary to dispose fecal matter, including honey bucket wastes, properly at any time of the year, in order to prevent the possible outbreak of diseases caused by enteric microorganisms.

Chapter Five

Conclusions and future work

5.1. Conclusions

This study revealed that fecal matter mixed with soil at different moisture levels is capable of supporting total coliforms for an extended period of time, at a wide range of temperatures. Soil samples placed at different temperatures for at least 170 days had a large fraction of viable coliforms present in them. At room temperature, the moisture content in the soil, varying from 24% to 49%, had no significant influence on the die-off rates of total coliforms, but die-off rates were higher at this temperature than for the samples placed at subzero temperatures. Even at subzero temperatures, a general trend of decrease in die-off rate with decrease in temperature was observed. Samples placed at uncontrolled temperatures experienced subzero temperature for most of the experiment's durations but still showed faster die-off rates than many samples placed at controlled subzero temperatures. At subzero temperatures, die-off rates increased with an increase in the moisture content in the soil.

Survival of total coliforms in the soil samples at a wide range of temperatures and moistures indicates the possibility of other enteric microorganisms also being able to survive in such environments. In rural Alaska, open dumps are used for disposal of honey buckets. Honey bucket wastes are not covered or incinerated for a long period and may get mixed with the soil. In winter the soil surface and honey buckets are covered with snow and the possibility of transfer of enteric microorganisms from a honey bucket is low.

In the thawing season, enteric microorganisms may get transported along with surface runoff and contaminate water sources. Vehicles, people, and animals commuting from the dumpsites are also likely to transmit enteric microorganisms into the communities and pose a health threat. This study suggests that it is necessary to dispose fecal wastes from any sources at proper places, where they are treated, so that possibilities of release of enteric organisms into the environment and associated health threats are minimized.

5.2. Future work

The results of this research provided evidence of long term survival of coliform bacteria in soil at different moisture levels at different subzero temperatures. The moisture content in the soil was found to be one of the factors which affect the survivability of the coliform bacteria. Other researchers have suggested that the survivability of the coliform bacteria depends upon the type of soil into which it is introduced. The soil used in this study was only silty loam. Constant pre-freezing moisture levels were maintained in the soil samples used in this study. However, in the open environment, moisture level in soil may not remain constant for a long time. Soil in different places may also vary in characteristics other than moisture. pH of the soil and exposure to solar radiation were not accounted for in this study. Therefore, studying survivability of coliform bacteria in soil in the open environment at subzero temperatures should be the next step in this research.

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Appendix A

Particle size analysis

A.1. Hydrometer analysis

Temperature of water	= 22°C	Specific gravity of soil	= 2.756
Upper meniscus reading	= 5	Sp. gr. correction factor	= 0.981
Lower meniscus reading	= 6	Value of K	= 0.01292
Zero correction (F_Z)	= 5	Temperature correction (F_T)	= 0.65
Meniscus correction	= 1	Dry mass of the soil	= 50.90 g

Elapsed Time t (min)	Average Hydrometer Reading	Composite Correction (F) = $F_M + F_T - F_Z$	Corrected Hydrometer Reading R_c	Effective Length L (cm)	Diameter of Soil Particle D (mm)	Percent Finer F (%)
1	40.00	-3.35	36.65	10.3	0.0415	70.6
2	30.00	-3.35	26.65	12.0	0.0316	51.4
4	24.00	-3.35	20.65	13.0	0.0232	39.8
8	16.50	-3.35	13.15	14.2	0.0172	25.3
15	14.00	-3.35	10.65	14.6	0.0127	20.5
30	11.50	-3.35	8.15	15.0	0.0091	15.7
60	11.00	-3.35	7.65	15.1	0.0065	14.7
120	10.50	-3.35	7.15	15.2	0.0046	13.8
240	10.00	-3.35	6.65	15.3	0.0033	12.8
480	9.75	-3.35	6.40	15.3	0.0023	12.3
1440	9.00	-3.35	5.65	15.4	0.0013	10.9

A.2. Sieve analysis

Total soil mass = 800 g

Sieve No.	Sieve Size (mm)	Mass of empty sieve (g)	Mass of Sieve + Soil (g)	Mass of Soil (g)	Cumulative Mass (g)	Mass Passing (g)	Percent Finer F (%)
4	4.750	817.0	817.2	0.2	0.2	799.8	100.0
10	2.000	482.9	484.9	2.0	2.2	797.8	99.7
30	0.595	472.4	508.3	35.9	38.1	761.9	95.2
60	0.250	360.4	437.1	76.7	114.8	685.2	85.7
100	0.149	353.6	387.3	33.7	148.5	651.5	81.4
200	0.750	302.8	358.6	55.8	204.3	595.7	74.5
Pan		467.8	1062.8	595.0	799.3	0.0	0.0

Appendix B

Total coliform survivability data obtained from the study and modeling at Different temperatures and moisture conditions

B.1. Total coliforms in the soil with 24% moisture and placed at room temperature.

Number of days samples placed at room temp.	Log of MPN values of coliform population in the samples			Standard deviation of Log MPN (σ)	95% confidence intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% confidence. interval limit about the best fit line	
0	7.69	7.52	6.69	0.53	1.05	7.30	7.36	0.004	6.75	7.97
20	7.04	6.66	5.69	0.70	1.37	6.46	7.00	0.289	6.49	7.52
36	7.69	6.32	6.36	0.20	0.40	6.46	6.72	0.067	6.26	7.17
58	6.90	6.41	6.52	0.25	0.50	6.61	6.32	0.083	5.94	6.71
78	6.52	6.34	6.34	0.10	0.20	6.40	5.97	0.190	5.61	6.32
99	6.04	6.11	5.69	0.23	0.44	5.95	5.59	0.129	5.23	5.95
130	5.11	5.36	5.52	0.20	0.40	5.33	5.04	0.088	4.60	5.47
158	4.90	4.36	4.90	0.31	0.61	4.72	4.53	0.034	3.99	5.08
199	3.11	3.54	3.36	0.14	0.27	3.27	3.98	0.501	3.29	4.68
Σ=768						Σ= 52.51		Σ= 1.383		

Slope = -0.018
Unbiased estimator of std. dev. = 0.198

Intercept = 7.360
95% confidence interval limits of slope = -0.024 and -0.012

B.2. Total coliforms in the soil with 24% moisture and placed at -5°C.

Number of days samples placed at -5°C	Log of MPN values of coliform population in the samples			Standard deviation of Log MPN (σ)	95% confidence intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% confidence interval limit about the best fit line	
0	7.69	7.52	6.69	0.53	1.05	7.30	7.32	0.000	7.07	7.58
20	7.38	7.49	7.73	0.18	0.35	7.53	7.29	0.058	7.08	7.51
47	7.11	6.90	7.54	0.33	0.64	7.19	7.26	0.005	7.08	7.43
60	6.90	7.04	7.38	0.25	0.49	7.11	7.24	0.018	7.08	7.40
81	7.11	6.90	6.97	0.11	0.22	6.99	7.21	0.046	7.07	7.36
100	7.38	7.34	7.34	0.02	0.04	7.36	7.18	0.029	7.04	7.33
131	7.23	7.38	7.11	0.13	0.26	7.24	7.14	0.010	6.96	7.32
160	6.96	6.96	6.73	0.13	0.26	6.89	7.10	0.046	6.88	7.33
193	7.20	7.20	7.20	0.00	0.00	7.20	7.06	0.022	6.77	7.35
Σ=792						Σ= 0.235		Σ= 1.189		

Slope = -0.001
Unbiased estimator of std. dev. = 0.034

Intercept = 7.322
95% confidence interval limits of slope = -0.004 and 0.001

B.3. Total coliforms in the soil with 24% moisture and placed at -15°C.

Number of days samples placed at -15°C	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	7.69	7.52	6.69	0.53	1.05	7.30	7.32	0.000	7.07	7.58
20	7.38	7.49	7.73	0.18	0.35	7.53	7.29	0.058	7.08	7.51
47	7.11	6.90	7.54	0.33	0.64	7.19	7.26	0.005	7.08	7.43
60	6.90	7.04	7.38	0.25	0.49	7.11	7.24	0.018	7.08	7.40
81	7.11	6.90	6.97	0.11	0.22	6.99	7.21	0.046	7.07	7.36
100	7.38	7.34	7.34	0.02	0.04	7.36	7.18	0.029	7.04	7.33
131	7.23	7.38	7.11	0.13	0.26	7.24	7.14	0.010	6.96	7.32
160	6.96	6.96	6.73	0.13	0.26	6.89	7.10	0.046	6.88	7.33
193	7.20	7.20	7.20	0.00	0.00	7.20	7.06	0.022	6.77	7.35
Σ=792						Σ= 64.81		Σ=0.235		

Slope = -0.018
Unbiased estimator of std. dev. = 0.198

Intercept = 7.360
95% confidence interval limits of slope = -0.024 and -0.012

B.4. Total coliforms in the soil with 24% moisture and placed at -20°C.

Number of days samples placed at -20°C	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	7.69	7.52	6.69	0.53	1.05	7.30	7.36	0.004	7.18	7.55
30	7.73	7.73	7.11	0.36	0.70	7.53	7.35	0.032	7.20	7.49
54	7.38	7.34	7.11	0.14	0.28	7.28	7.33	0.003	7.22	7.45
77	7.34	7.38	7.38	0.02	0.04	7.37	7.32	0.002	7.22	7.42
98	7.23	7.38	7.04	0.17	0.33	7.22	7.31	0.008	7.21	7.40
130	7.38	7.54	7.11	0.22	0.43	7.35	7.29	0.003	7.19	7.40
147	7.38	7.11	6.90	0.24	0.47	7.13	7.28	0.023	7.16	7.40
168	7.11	7.38	7.11	0.15	0.30	7.20	7.27	0.004	7.13	7.41
196	7.54	7.54	7.11	0.25	0.49	7.40	7.25	0.022	7.08	7.43
Σ=900						Σ= 65.77		Σ=0.102		

Slope = -0.00057
Unbiased estimator of std. dev. = 0.015

Intercept = 7.365
95% confidence interval limits of slope = -0.002 and 0.001

B.5. Total coliforms in the soil with 24% moisture and placed at -28°C.

Number of days samples placed at -28°C	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	7.69	7.52	6.69	0.53	1.05	7.30	7.47	0.028	7.19	7.74
26	7.73	7.54	7.38	0.18	0.35	7.55	7.43	0.014	7.21	7.66
49	7.73	7.11	7.34	0.31	0.61	7.40	7.40	0.000	7.22	7.59
67	7.38	6.90	7.23	0.25	0.48	7.17	7.38	0.043	7.22	7.54
84	7.34	7.38	7.11	0.14	0.28	7.28	7.35	0.006	7.21	7.50
98	7.54	7.96	7.73	0.21	0.41	7.75	7.34	0.169	7.19	7.48
121	7.38	7.73	7.38	0.20	0.40	7.50	7.30	0.037	7.15	7.46
146	7.38	7.11	7.11	0.15	0.30	7.20	7.27	0.005	7.08	7.46
171	7.04	7.11	7.23	0.10	0.19	7.13	7.24	0.012	7.00	7.47
197	7.26	7.04	7.04	0.12	0.24	7.11	7.20	0.008	6.92	7.49
Σ=959						Σ= 73.38		Σ=0.322		

Slope = -0.001
Unbiased estimator of std. dev. = 0.040

Intercept = 7.468
95% confidence interval limits of slope = -0.004 and 0.001

B.6. Total coliforms in the soil with 37% moisture and placed at room temperature.

Number of days samples placed at room temp.	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	7.54	7.38	7.73	0.18	0.35	7.55	7.31	0.057	7.31	7.31
16	7.54	7.23	7.38	0.16	0.31	7.38	7.08	0.094	7.08	7.08
39	6.85	6.52	6.69	0.16	0.32	6.68	6.74	0.003	6.74	6.74
53	6.36	6.69	6.36	0.19	0.37	6.47	6.53	0.004	6.53	6.53
77	5.95	6.52	5.78	0.39	0.76	6.08	6.18	0.010	6.18	6.18
104	5.04	4.90	4.90	0.08	0.16	4.95	5.79	0.706	5.79	5.79
125	5.23	5.15	5.54	0.21	0.41	5.31	5.48	0.029	5.48	5.48
144	5.45	5.73	4.52	0.63	1.24	5.23	5.20	0.001	5.20	5.20
168	5.15	5.73	5.23	0.32	0.62	5.37	4.85	0.274	4.85	4.85
193	4.43	4.90	4.69	0.23	0.46	4.67	4.48	0.038	4.48	4.48
215	4.04	4.11	3.90	0.11	0.22	4.02	4.16	0.019	4.16	4.16
238	3.73	3.96	3.96	0.13	0.26	3.89	3.82	0.005	3.82	3.82
Σ=1372						Σ= 67.61		Σ=1.24		

Slope = -0.015
Unbiased estimator of std. dev. = 0.124

Intercept = 7.313
95% confidence interval limits of slope = -0.018 and -0.012

B.7. Total coliforms in the soil with 37% moisture and placed at -5°C.

Number of days samples placed at -5°C	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	7.54	7.38	7.73	0.18	0.35	7.55	7.17	0.149	7.17	7.17
16	6.90	7.11	6.69	0.21	0.42	6.90	7.08	0.032	7.08	7.08
38	6.90	6.90	6.66	0.14	0.27	6.82	6.96	0.021	6.96	6.96
53	6.69	6.85	7.04	0.18	0.34	6.86	6.88	0.001	6.88	6.88
69	6.69	6.36	6.90	0.27	0.53	6.65	6.80	0.022	6.80	6.80
87	6.52	6.69	6.69	0.10	0.19	6.63	6.70	0.005	6.70	6.70
114	6.90	7.11	7.34	0.22	0.44	7.12	6.56	0.315	6.56	6.56
139	5.69	6.38	5.90	0.35	0.69	5.99	6.42	0.188	6.42	6.42
168	5.90	6.38	6.11	0.24	0.47	6.13	6.27	0.019	6.27	6.27
191	5.90	6.11	6.04	0.11	0.22	6.02	6.14	0.016	6.14	6.14
214	6.11	6.38	6.23	0.13	0.26	6.24	6.02	0.048	6.02	6.02
241	5.90	6.11	5.90	0.12	0.24	5.97	5.88	0.008	5.88	5.88
Σ=1330						Σ= 78.88		Σ=0.824		

Slope = -0.005
Intercept = 7.166
Unbiased estimator of std. dev. = 0.082
95% confidence interval limits of slope = -0.008 and -0.003

B.8. Total coliforms in the soil with 37% moisture and placed at -15°C.

Number of days samples placed at -15°C	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	7.54	7.38	7.73	0.18	0.35	7.55	7.12	0.186	6.72	7.52
6	6.90	7.04	7.38	0.25	0.49	7.11	7.09	0.000	6.70	7.48
17	6.90	6.52	6.36	0.28	0.54	6.59	7.04	0.203	6.68	7.41
32	6.36	7.11	6.52	0.40	0.78	6.66	6.97	0.095	6.72	7.31
73	7.54	7.73	7.73	0.11	0.21	7.67	6.78	0.784	6.51	7.05
88	6.52	6.69	6.69	0.10	0.19	6.63	6.71	0.007	6.46	6.97
114	6.11	5.69	6.11	0.24	0.48	5.97	6.59	0.387	6.36	6.83
139	6.52	6.69	6.23	0.23	0.46	6.48	6.48	0.000	6.23	6.72
168	6.34	6.23	6.40	0.09	0.17	6.32	6.35	0.000	6.07	6.62
192	6.38	6.23	6.11	0.13	0.26	6.24	6.23	0.000	5.92	6.55
215	6.38	6.11	6.11	0.15	0.30	6.20	6.13	0.006	5.77	6.48
242	6.54	5.90	5.90	0.37	0.73	6.11	6.00	0.012	5.59	6.42
258	5.74	5.73	6.20	0.27	0.53	5.89	5.93	0.001	5.48	6.38
Σ=1544						Σ= 85.44		Σ=1.681		

Slope = -0.005
Intercept = 7.121
Unbiased estimator of std. dev. = 0.153
95% confidence interval limits of slope = -0.007 and -0.002

B.9. Total coliforms in the soil with 37% moisture and placed at -20°C.

Number of days samples placed at -20°C	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	7.54	7.38	7.73	0.18	0.35	7.55	7.14	0.172	6.92	7.36
4	7.23	7.11	7.11	0.07	0.13	7.15	7.12	0.001	6.91	7.34
6	7.54	7.11	7.54	0.25	0.49	7.40	7.12	0.080	6.90	7.33
17	7.11	6.85	-	0.19	0.37	6.98	7.08	0.010	6.93	7.28
30	6.90	6.69	6.69	0.12	0.23	6.76	7.04	0.077	6.85	7.22
47	-	7.15	7.15	0.00	0.00	7.15	6.98	0.028	6.81	7.15
66	6.69	6.36	6.52	0.16	0.32	6.52	6.92	0.154	6.76	7.07
92	6.36	6.69	6.52	0.16	0.32	6.52	6.83	0.093	6.68	6.98
120	6.96	6.73	6.54	0.21	0.41	6.75	6.74	0.000	6.58	6.89
143	6.73	6.73	6.54	0.11	0.21	6.67	6.66	0.000	6.49	6.82
174	6.54	6.73	6.38	0.18	0.35	6.55	6.55	0.000	6.36	6.75
203	6.23	6.38	6.38	0.09	0.17	6.33	6.46	0.016	6.23	6.68
233	6.23	6.54	6.11	0.22	0.44	6.30	6.36	0.004	6.09	6.62
262	6.54	6.73	6.54	0.11	0.21	6.61	6.26	0.121	5.95	6.57
Σ=1397						Σ= 95.24		Σ=0.756		

Slope = -0.003
Unbiased estimator of std. dev. = 0.063

Intercept = 7.138
95% confidence interval limits of slope = -0.005 and -0.002

B.10. Total coliforms in the soil with 37% moisture and placed at -28°C.

Number of days samples placed at -28°C	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	7.54	7.38	7.73	0.18	0.35	7.55	7.31	0.057	7.14	7.49
4	7.54	7.96	7.54	0.24	0.47	7.68	7.31	0.143	7.14	7.47
6	7.11	7.38	6.90	0.24	0.47	7.13	7.30	0.029	7.14	7.47
14	7.38	7.38	7.11	0.15	0.30	7.29	7.28	0.000	7.17	7.44
30	7.38	6.36	6.90	0.51	1.00	6.88	7.25	0.136	7.11	7.39
47	7.15	7.15	-	0.00	0.00	7.15	7.21	0.004	7.08	7.34
71	7.15	6.90	7.23	0.17	0.34	7.09	7.16	0.005	7.04	7.28
94	7.11	7.11	7.38	0.15	0.30	7.20	7.11	0.009	7.00	7.22
116	7.38	7.04	6.52	0.43	0.85	6.98	7.06	0.007	6.95	7.18
139	6.69	7.04	6.97	0.19	0.36	6.90	7.01	0.012	6.89	7.14
165	6.90	7.11	6.69	0.21	0.42	6.90	6.95	0.003	6.81	7.10
196	7.11	7.23	6.90	0.17	0.33	7.08	6.89	0.037	6.72	7.06
226	6.96	6.54	6.73	0.21	0.41	6.75	6.82	0.006	6.62	7.02
263	6.73	6.54	7.20	0.34	0.67	6.83	6.74	0.007	6.50	6.99
Σ=1371						Σ= 99.41		Σ=0.448		

Slope = -0.002
Unbiased estimator of std. dev. = 0.037

Intercept = 7.314
95% confidence interval limits of slope = -0.003 and -0.001

B.11. Total coliform in the soil with 49% moisture and placed at room temperature.

Number of days samples placed at room temp.	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	8.20	8.20	7.54	0.38	0.75	7.98	8.06	0.006	7.80	8.31
20	7.96	7.73	7.45	0.26	0.51	7.71	7.76	0.002	7.55	7.98
40	7.73	7.97	7.54	0.21	0.42	7.75	7.46	0.081	7.29	7.64
59	6.69	7.11	7.04	0.23	0.44	6.95	7.18	0.055	7.03	7.34
79	6.90	6.90	6.52	0.00	0.00	6.86	6.89	0.001	6.74	7.03
100	6.52	6.52	6.69	0.10	0.19	6.58	6.57	0.000	6.42	6.72
126	6.36	6.36	6.52	0.09	0.18	6.41	6.19	0.051	6.01	6.37
148	5.90	5.69	6.11	0.21	0.42	5.90	5.86	0.002	5.64	6.08
170	5.36	5.36	5.36	0.00	0.00	5.36	5.53	0.030	5.27	5.80
Σ=742						Σ= 61.51		Σ=0.226		

Slope = -0.015
Unbiased estimator of std. dev. = 0.032

Intercept = 8.058
95% confidence interval limits of slope = -0.017 and -0.012

B.12. Total coliform in the soil with 49% moisture and placed at -5°C.

Number of days samples placed at -5°C	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	8.20	8.20	7.54	0.38	0.75	7.98	7.43	0.309	6.47	8.38
24	7.34	7.38	7.54	0.11	0.21	7.42	7.16	0.067	6.38	7.94
45	6.69	6.90	7.38	0.35	0.69	6.99	6.93	0.003	6.28	7.58
71	5.52	5.11	5.52	0.23	0.46	5.38	6.65	1.592	6.10	7.20
95	6.04	6.11	6.38	0.18	0.35	6.18	6.38	0.041	5.84	6.92
120	6.11	6.11	5.69	0.24	0.48	5.97	6.11	0.018	5.48	6.73
146	6.11	6.11	6.38	0.15	0.30	6.20	5.82	0.147	5.03	6.60
176	5.90	5.69	5.90	0.12	0.23	5.83	5.49	0.116	4.48	6.50
Σ=677						Σ= 51.96		Σ=2.293		

Slope = -0.011
Unbiased estimator of std. dev. = 0.382

Intercept = 7.428
95% confidence interval limits of slope = -0.020 and -0.002

B.13. Total coliform in the soil with 49% moisture and placed at -15°C.

Number of days samples placed at -15°C	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	8.20	8.20	7.54	0.38	0.75	7.98	7.30	0.468	7.30	7.30
19	7.04	6.52	6.52	0.30	0.59	6.69	7.19	0.243	7.19	7.19
43	7.11	6.69	6.85	0.21	0.42	6.88	7.04	0.025	7.04	7.04
65	6.69	6.52	6.90	0.19	0.37	6.70	6.91	0.043	6.91	6.91
94	6.73	6.73	6.54	0.11	0.21	6.67	6.73	0.004	6.73	6.73
125	6.90	6.69	6.36	0.27	0.53	6.65	6.55	0.010	6.55	6.55
153	6.73	6.73	5.90	0.48	0.94	6.45	6.38	0.006	6.38	6.38
186	6.23	6.11	6.38	0.13	0.26	6.24	6.18	0.004	6.18	6.18
Σ=685						Σ= 54.28		Σ=0.803		

Slope = -0.006
Unbiased estimator of std. dev. = 0.134

Intercept = 7.300
95% confidence interval limits of slope = -0.011 and -0.001

B.14. Total coliform in the soil with 49% moisture and placed at -20°C.

Number of days samples placed at -20°C	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	8.20	8.20	7.54	0.38	0.75	7.98	7.70	0.080	7.31	8.09
29	7.73	7.96	7.11	0.44	0.86	7.60	7.47	0.018	7.16	7.77
53	7.34	6.90	7.11	0.22	0.44	7.12	7.28	0.025	7.03	7.53
76	7.38	6.90	6.52	0.43	0.85	6.93	7.09	0.026	6.87	7.31
94	6.73	6.73	6.54	0.11	0.21	6.67	6.95	0.077	6.73	7.16
125	6.54	6.38	6.73	0.18	0.35	6.55	6.70	0.022	6.45	6.95
156	6.38	6.54	6.38	0.09	0.19	6.43	6.45	0.000	6.13	6.77
186	6.73	6.54	6.38	0.18	0.35	6.55	6.21	0.117	5.80	6.62
Σ=719						Σ= 55.85		Σ=0.365		

Slope = -0.008
Unbiased estimator of std. dev. = 0.061

Intercept = 7.701
95% confidence interval limits of slope = -0.012 and -004

B.15. Total coliform in the soil with 49% moisture and placed at -28°C.

Number of days samples placed at -28°C	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	8.20	8.20	7.54	0.38	0.75	7.98	7.50	0.236	7.02	7.98
31	6.90	7.23	7.11	0.17	0.33	7.08	7.38	0.087	7.00	7.75
51	7.23	6.90	6.52	0.36	0.70	6.88	7.30	0.171	6.98	7.62
74	7.96	6.69	7.54	0.65	1.27	7.40	7.20	0.038	6.93	7.48
97	7.11	7.04	6.69	0.23	0.44	6.95	7.11	0.027	6.85	7.38
129	7.38	6.90	6.90	0.28	0.55	7.06	6.99	0.005	6.68	7.30
159	7.11	6.69	6.90	0.21	0.42	6.90	6.87	0.001	6.47	7.26
196	6.73	6.73	6.96	0.13	0.26	6.81	6.72	0.008	6.20	7.24
Σ=737						Σ= 57.06		Σ=573		

Slope = -0.004
Unbiased estimator of std. dev. = 0.096

Intercept = 7.498
95% confidence interval limits of slope = -0.008 and 0.000

B.16. Total coliform in the soil with 24% moisture and placed at uncontrolled temperature.

Number of days samples placed at uncontrolled temp.	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	7.69	7.52	6.69	0.53	0.61	7.30	7.25	0.003	6.99	7.51
8	6.85	6.90	7.15	0.16	0.18	6.96	7.17	0.044	6.93	7.42
28	7.04	7.11	7.23	0.10	0.11	7.13	6.99	0.018	6.79	7.20
35	6.90	6.52	6.90	0.22	0.25	6.77	6.93	0.025	6.78	7.12
45	7.04	7.23	5.69	0.84	0.95	6.65	6.84	0.034	6.66	7.02
59	6.69	6.90	6.66	0.13	0.15	6.75	6.71	0.001	6.55	6.88
73	6.90	6.85	6.66	0.12	0.14	6.80	6.59	0.047	6.43	6.74
91	6.45	6.73	6.96	0.26	0.29	6.71	6.42	0.085	6.27	6.58
111	6.96	6.73	6.34	0.31	0.36	6.68	6.24	0.191	6.08	6.41
129	5.90	5.52	5.69	0.19	0.21	5.70	6.08	0.143	5.89	6.27
145	5.90	5.69	5.36	0.27	0.31	5.65	5.94	0.081	5.72	6.15
169	5.90	5.90	5.36	0.31	0.35	5.72	5.72	0.000	5.45	5.98
192		5.38	5.73	0.25	0.35	5.56	5.51	0.002	5.20	5.82
Σ=1085						Σ= 84.39		Σ=0.675		

Slope = -0.009
Unbiased estimator of std. dev. = 0.061

Intercept = 7.246
95% confidence interval limits of slope = -0.012 and 0.006

B.17. Total coliform in the soil with 37% moisture and placed at uncontrolled temperature.

Number of days samples placed at uncontrolled temp.	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	6.36	6.69	6.36	0.19	0.21	6.47	6.15	0.100	5.57	6.74
8	6.90	6.52	6.69	0.19	0.21	6.70	6.06	0.415	5.52	6.60
28	6.43	6.52	6.52	0.05	0.06	6.49	5.82	0.453	5.36	6.27
35	5.23	5.66	6.11	0.44	0.50	5.67	5.73	0.004	5.40	6.16
45	5.36	5.36	5.04	0.18	0.21	5.25	5.61	0.127	5.22	6.00
59	4.90	4.36	4.90	0.31	0.35	4.72	5.44	0.522	5.09	5.80
73	4.66	4.69	4.43	0.14	0.16	4.59	5.27	0.459	4.94	5.61
91	4.23	4.69	4.23	0.27	0.30	4.38	5.05	0.450	4.72	5.39
111	4.52	4.36	4.36	0.09	0.10	4.41	4.81	0.159	4.44	5.19
119	4.54	4.96	4.73	0.21	0.24	4.75	4.72	0.001	4.31	5.12
135	4.96	4.96	5.20	0.14	0.16	5.04	4.52	0.271	4.06	4.99
157	4.73	4.73	4.54	0.11	0.12	4.67	4.26	0.170	3.69	4.82
182	3.85	4.45	4.45	0.35	0.39	4.25	3.96	0.085	3.27	4.65
Σ=1043						Σ= 67.40		Σ=3.217		

Slope = -0.012
Unbiased estimator of std. dev. = 0.292

Intercept = 6.155
95% confidence interval limits of slope = -0.018 and -0.006

B.18. Total coliform in the soil with 49% moisture and placed at uncontrolled temperature.

Number of days samples placed at uncontrolled temp.	Log of MPN values of coliform population in the samples			Std. dev. of Log MPN (σ)	95% conf. intervals for the samples	Mean of log MPN	MPN values in best fit line	Square of residuals	95% conf. interval limit about the best fit line	
0	8.20	8.20	7.54	0.38	0.43	7.98	7.30	0.475	6.74	7.85
8	7.96	7.73	7.54	0.21	0.24	7.75	7.13	0.380	6.61	7.65
28	6.66	6.52	6.52	0.08	0.09	6.57	6.72	0.023	6.27	7.16
34	6.69	6.90	6.90	0.12	0.14	6.83	6.59	0.055	6.27	7.02
45	5.69	5.52	6.36	0.45	0.50	5.86	6.37	0.261	5.98	6.75
59	4.90	4.90	5.34	0.25	0.29	5.05	6.08	1.059	5.73	6.43
73	5.11	5.54	5.38	0.22	0.25	5.35	5.79	0.197	5.46	6.12
91	4.97	4.97	5.34	0.21	0.24	5.10	5.42	0.104	5.09	5.75
111	4.90	4.90	4.90	0.00	0.00	4.90	5.01	0.012	4.65	5.37
129	4.90	5.11	4.52	0.30	0.34	4.84	4.64	0.043	4.23	5.05
145	4.69	4.90	4.90	0.12	0.14	4.83	4.31	0.273	3.84	4.77
169	4.34	4.36	4.11	0.14	0.16	4.27	3.81	0.213	3.24	4.38
192	3.11	3.23		0.08	0.11	3.17	3.34	0.027	2.66	4.01
Σ=1084						Σ= 72.49		Σ=3.123		

Slope = -0.021
Unbiased estimator of std. dev. = 0.284

Intercept = 7.295
95% confidence interval limits of slope = -0.026 and -0.015